Form Approved. O.M.B. No. 2070-0012. Approval Expires 10-31-96.

U.S. ENVIRONMENTAL PROTECTION AGENCY

PREMANUFACTURE NOTICE

AGENCY USE ONLY

Date of receipt

RECEIVED
OPPT CBIC

08 DEC 22 AM 6:05

Company Sanitized

Enter the total number of pages in the Premanufacture Notice ▶ 580

Document control number (DCN) EPA case number P-09-135

GENERAL INSTRUCTIONS

TS - A D 0 0 0 2

- You must provide all information requested in this form to the extent that it is known to or reasonably ascertainable by you. Make reasonable estimates if you do not have actual data.
- Before you complete this form, you should read the "Instructions Manual for Premanufacture Notification" (the Instructions Manual is available from the Toxic Substances Control Act (TSCA) Information Service by calling 202-554-1404, or faxing 202-554-5603).
- If a user fee has been remitted for this notice (40 CFR 700.45), indicate in the boxes above the TS-user fee identification number you have generated. Remember, your user fee ID number must also appear on your corresponding fee remittance, which is sent to EPA, Washington Financial Management Center (3303), P.O. 360399M, Pittsburgh, PA 15251-6399, Attn. TSCA User fee.

Part I — GENERAL INFORMATION

You must provide the currently correct Chemical Abstracts (CA) Name of the new chemical substance, even if you claim the identity as confidential. You may authorize another person to submit chemical identity information for you, but your submission will not be complete and the review will not begin until EPA receives this information. A letter in support of your submission should reference your TS user fee identification number. You must submit an original and two copies of this notice including all test data. If you claimed any information as confidential, a single sanitized copy must also be submitted.

Part II — HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE

If there are several manufacture, processing, or use operations to be described in Part II, sections A and B of this notice, reproduce the sections as needed.

Part III — LIST OF ATTACHMENTS

Attach additional sheets if there is not enough space to answer a question fully. Label each continuation sheet with the corresponding section heading. In Part III, list these attachments, any test data or other data and any optional information included in the notice.

OPTIONAL INFORMATION

You may include any information that you want EPA to consider in evaluating the new substance. On page 11 of this form, space has been provided for you to describe pollution prevention and recycling information you may have regarding the new substance.

So-called "binding" boxes are included throughout this form for you to indicate your willingness to be bound to certain statements you make in this section, such as use, production volume, protective equipment . . . This option is intended to reduce delays that routinely accompany the development of consent orders or Significant New Use Rules. Except in the case of exemption applications (such as TMEA, LVE, LOREX) where certain information provided in such notification is binding on the submitter when the Agency approves the exemption application, checking a binding box in this notice does not by itself prohibit the submitter from later deviating from the information (except chemical identity) reported in the form.

CONFIDENTIALITY CLAIMS

You may claim any information in this notice as confidential. To assert a claim on the form, mark (X) the confidential box next to the information that you claim as confidential. To assert a claim in an attachment, circle or bracket the information you claim as confidential. If you claim information in the notices as confidential, you must also provide a sanitized version of the notice, (including attachments). For additional instructions on claiming information as confidential, read the Instructions Manual.

☒ Mark (x) if any information in this notice is claimed as confidential.

TEST DATA AND OTHER DATA

You are required to submit all test data in your possession or control and to provide a description of all other data known to or reasonably ascertainable by you, if these data are related to the health and environmental effects on the manufacture, processing, distribution in commerce, use, or disposal of the new chemical substance. Standard literature citations may be submitted for data in the open scientific literature. Complete test data (written in English), not summaries of data, must be submitted if they do not appear in the open literature. You should clearly identify whether test data is on the substance or on an analog. Also, the chemical composition of the tested material should be characterized. Following are examples of test data and other data. Data should be submitted according to the requirements of §720.50 of the Premanufacture Notification Rule (40 CFR Part 720).

Test Data (Check Below any included in this notice)

• Environmental fate data	☒	Yes	• Other data	☐	Yes
• Health effects data	☒	Yes	Risk assessments	☐	
• Environmental effects data	☐	Yes	Structure/activity relationships	☐	
• Physical/Chemical Properties*	☒	Yes	Test data not in the possession or control of the submitter	☐	

* A physical and chemical properties worksheet is located on the last page of this form.

TYPE OF NOTICE (Check Only One)

- ☒ PMN (Premanufacture Notice)
- ☐ INTERMEDIATE PMN (submitted in sequence with final product PMN)
- ☐ SNUN (Significant New Use Notice)
- ☐ TMEA (Test Marketing Exemption Application)
- ☐ LVE (Low Volume Exemption) @ 40 CFR 723.50(c)(1)
- ☐ LOREX (Low Release/Low Exposure Exemption) @ 40 CFR 723.50(c)(2)
- ☐ LVE Modification ☐ LOREX Modification

IS THIS A CONSOLIDATED PMN? ☐ Yes

# of chemicals or polymers 1
(Prenotice Communication # required, enter # on page 3)

315899

CERTIFICATION -- A Printed copy of this signature page, with original signature, must be submitted

I certify that to the best of my knowledge and belief:

1. The company named in Part I, section A, subsection 1a of this notice form intends to manufacture or import for a commercial purpose, other than in small quantities solely for research and development, the substance identified in Part I, Section B.
2. All information provided in this notice is complete and truthful as of the date of submission.
3. I am submitting with this notice all test data in my possession or control and a description of all other data known to or reasonably ascertainable by me as required by §720.50 of the Premanufacture Notification Rule.

Additional Certification Statements:

If you are submitting a PMN, Intermediate PMN, Consolidated PMN, or SNUN, check the following **user fee** certification statement that applies:

- [x] The Company named in Part I, Section A has remitted the fee of $2500 specified in 40 CFR 700.45(b), or
- [ ] The Company named in Part I, Section A has remitted the fee of $1000 for an Intermediate PMN (defined @ 40 CFR 700.43) in accordance with 40 CFR 700.45(b), or
- [ ] The Company named in Part I Section A is a small business concern under 40 CFR 700.43 and has remitted a fee of $100 in accordance with 40 CFR 700.45(b).

If you are submitting a **low volume exemption (LVE)** application in accordance with 40 CFR 723.50(c)(1) or a **Low release and low exposure exemption (LoRex)** application in accordance with 40 CFR 723.50(c)(2), check the following certification statements:

- [ ] The manufacturer submitting this notice intends to manufacture or import the new chemical substance for commercial purposes, other than in small quantities solely for research and development, under the terms of 40 CFR 723.50.
- [ ] The manufacturer is familiar with the terms of this section and will comply with those terms; and
- [ ] The new chemical substance for which the notice is submitted meets all applicable exemption conditions.
- [ ] If this application is for an LVE in accordance with 40 CFR 723.50(c)(1), the manufacturer intends to commence manufacture of the exempted substance for commercial purposes within 1 year of the date of the expiration of the 30 day review period.

The accuracy of the statements you make in this notice should reflect your best prediction of the anticipated facts regarding the chemical substance described herein. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 USC 1001.

Signature and title of Authorized Official (Original Signature Required)	Date	Confidential
Andy Wang Regulatory Affairs Manager	12/18/2008	☐
Signature of agent - (if applicable)	Date	☐

Part I -- GENERAL INFORMATION

Section A -- SUBMITTER IDENTIFICATION

Mark () the "Confidential" box next to any subsection you claim as confidential

Confi-dential

1a. Person Submitting Notice (in U.S.)

Name of authorized official: Andy Wang

Position: Regulatory Affairs Manager

Company: ICL-IP America Inc.

Mailing address (number and street): 95 MacCorkle Avenue, S.W.

City, State: South Charleston, WV

Postal Code: 25303

b. Agent (if applicable)

Name of authorized official:

Position:

Company:

Mailing address (number and street):

City, State:

Postal Code:

Telephone (include area code):

c. If you are submitting this notice as part of a joint submission, mark (X) this box. → ☐

Joint Submitter (if applicable)

Name of authorized official:

Position:

Company:

Mailing address (number and street):

City, State:

Province, Country:

Postal Code:

Telephone (include country or area code):

2. Technical Contact (in U.S.)

Name of authorized official: Andy Wang

Position: Regulatory Affairs Manager

Company: ICL-IP America Inc.

Mailing address (number and street): 420 Saw Mill River Road

City, State: Ardsley, NY

Postal Code: 10502

Telephone (include area code): (914)269-5928

3. If you have had a prenotice communication (PC) concerning this notice and EPA assigned a PC Number to the notice, enter the number. →		Mark (X) if none →	☐
4. If you previously submitted an exemption application for the chemical substance covered by this notice, enter the exemption number assigned by EPA. If you previously submitted a PMN for this substance enter the PMN number assigned by EPA (i.e. withdrawn or incomplete). →		Mark (X) if none →	☐
5. If you have submitted a notice of Bona fide intent to manufacture or import for the chemical substance covered by this notice, enter the notice number assigned by EPA. →		Mark (X) if none →	☐

6. Type of Notice - Mark (X)

1. ☐ Manufacture Only — ☐ Binding Option Mark (X)
2. ☒ Import Only — ☐ Binding Option Mark (X)
3. ☐ Both

Part I -- GENERAL INFORMATION -- Continued

Section B -- CHEMICAL IDENTITY INFORMATION: You must provide a currently correct Chemical Abstracts (CA) name of the substance based on the ninth Collective Index (9CI) of CA nomenclature rules and conventions.

Mark (X) the "Confidential" box next to any item you claim as confidential

Complete either item 1 (Class 1 or 2 substances) or 2 (Polymers) as appropriate. Complete all other items.

If another person will submit chemical identity information for you (for either Item 1 or 2), mark (X) the box at the right. Identify the name, company, and address of that person in a continuation sheet. → ☐

Confi-dential

1. Class 1 or 2 chemical substances (for definitions of class 1 and class 2 substances, see the Instructions Manual)

   a. Class of substance - Mark (X) ☐ Class 1 or ☒ Class 2

   b. Chemical name (Currently correct Chemical Abstracts (CA) Name that is consistent with TSCA Inventory listings for similar substances. For Class 1 substances a CA Index Name must be provided. For Class 2 substances either a CA Index Name or CA Preferred Name must be provided, which ever is appropriate based on CA 9CI nomenclature rules and conventions). ☒

   c. Please identify which method you used to develop or obtain the specified chemical identity information reported in this notice: (check one)
   ☒ Method 1 (CAS Inventory Expert Service - a copy of the Identification report obtained from the CAS Inventory Expert Services must be submitted as an attachment to this notice) ☐ Method 2 (Other Source)

   d. Molecular formula **CBI** ☐ CAS Registry Number (if a number already exists for the substance) ☐

   e. For a class 1 substance, provide a complete and correct chemical structure diagram. For a class 2 substance, provide a correct representative or partial chemical structure diagram, as complete as can be known, if one can be reasonably ascertained. Please see the E-PMN Instruction Manual for discussion of "native format" diagram software which can be helpful in reviewing your substance. ☐

☐ Mark (X) this box if you attach a continuation sheet.

For a class 2 substance - (1) List the immediate precursor substances with their respective CAS Registry Numbers. (2) Describe the nature of the reaction or process. (3) Indicate the range of composition and the typical composition (where appropriate).

e. (1) List the immediate precursor substances with their respective CAS Registry Numbers.
Name (CAS #)

Confi-dential: [X]

e. (2) Describe the nature of the reaction or process.

Confi-dential: [X]

e. (3) Indicate the range of composition and the typical composition (where appropriate).

Confi-dential: [ ]

[ ] Mark (X) this box if you attach a continuation sheet.

Part I -- GENERAL INFORMATION -- Continued

Section B -- CHEMICAL IDENTITY INFORMATION -- Continued

2. Polymers (For a definition of polymer, see the Instructions Manual.)

Confi-dential

a. Indicate the number-average weight of the lowest molecular weight composition of the polymer you intend to manufacture. Indicate maximum weight percent of low molecular weight species (not including residual monomers, reactants, or solvents) below 500 and below 1,000 absolute molecular weight of that composition. ☐

Describe the methods of measurement or the basis for your estimates: GPC ☐ Other ☐ : (Specify below)

(i) lowest number average molecular weight: _____

(ii) maximum weight % below 500 molecular weight: _____

(iii) maximum weight % below 1000 molecular weight: _____

☐ Mark (X) this box if you attach a continuation sheet.

b. You must make separate confidentiality claims for monomer or other reactant identity, composition information, and residual information. Mark (X) the "Confidential" box next to any item you claim as confidential

(1) - Provide the specific chemical name and CAS Registry Number (if a number exists) of each monomer or other reactant used in the manufacture of the polymer.
(2) - Mark (X) this column if entry in column (1) is confidential.
(3) - Indicate the typical weight percent of each monomer or other reactant in the polymer.
(4) - Choose "yes" from drop down menu if you want a monomer or other reactant used at two weight percent or less to be listed as part of the polymer description on the TSCA Chemical Substance Inventory.
(5) - Mark (X) this column if entries in columns (3) and (4) are confidential.
(6) - Indicate the maximum weight percent of each monomer or other reactant that may be present as a residual in the polymer as manufactured for commercial purposes.
(7) - Mark (X) this column if entry in column (6) is confidential.

Monomer or other reactant and CAS Registry Number (1)	Confi-dential (2)	Typical composition (3)	Include in identity (4)	Confi-dential (5)	Maximum residual (6)	Confi-dential (7)
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐
	☐	%	☐	☐	%	☐

☐ Mark (X) this box if you attach a continuation sheet.

	CBI
c. Please identify which method you used to develop or obtain the specified chemical identity information reported in this notice (check one). [x] Method 1 (CAS Inventory Expert Service - a copy of the identification report obtained from CAS Inventory Expert Service must be submitted as an attachment to this notice) [ ] Method 2 (other source)	[ ]
d. The currently correct Chemical Abstracts (CA) name for the polymer that is consistent with TSCA Inventory listings for similar polymers.	[ ]
CAS Registry Number (if a number already exists for the substance)	[ ]
e. Provide a correct representative or partial chemical structure diagram, as complete as can be known, if one can be reasonably ascertained. . Please see the E-PMN Instruction Manual for discussion of "native format" diagram software which can be helpful in reviewing your substance.	[ ]

[ ] Mark (X) this box if you attach a continuation sheet.

Part I -- GENERAL INFORMATION -- Continued

Section B -- CHEMICAL IDENTITY INFORMATION -- Continued

3. Impurities

(a) - Identify each impurity that may be reasonably anticipated to be present in the chemical substance as manufactured for commercial purpose. Provide the CAS Registry Number if available. If there are unidentified impurities, enter "unidentified."

(b) - Estimate the maximum weight % of each impurity. If there are unidentified impurities, estimate their total weight %.

Impurity and CAS Registry Number (a)	Maximum percent (b)	Confidential
	%	X
	%	X
	%	X
	%	X
	%	X
	%	X
	%	

☐ Mark (X) this box if you attach a continuation sheet.

4. Synonyms - Enter any chemical synonyms for the new chemical identified in subsection 1 or 2.

Confidential: X

☐ Mark (X) this box if you attach a continuation sheet.

5. Trade identification - List trade names for the new chemical substance identified in subsection 1 or 2.

Confidential: X

☐ Mark (X) this box if you attach a continuation sheet.

6. Generic chemical name - If you claim chemical identify as confidential, you must provide a generic name for your substance that reveals the specific chemical identity of the new chemical substance to the maximum extent possible. Refer to the TSCA Chemical Substance Inventory, 1985 Edition, Appendix B for guidance on developing generic names.

Pentabromobenzyl dialkylene glycol alkyl ether

☐ Mark (X) this box if you attach a continuation sheet.

7. Byproducts - Describe any byproducts resulting from the manufacture, processing, use, or disposal of the new chemical substance. Provide the CAS Registry Number if available.

Byproduct (1)	CAS Registry Number (2)	Confidential

☐ Mark (X) this box if you attach a continuation sheet.

Part I -- GENERAL INFORMATION -- Continued

Section C -- PRODUCTION, IMPORT, AND USE INFORMATION:

Mark (X) the "Confidential" box next to any item you claim as confidential.

1. **Production volume** -- Estimate the **maximum** production volume during the first 12 months of production. Also estimate the maximum production volume for any consecutive 12-month period during the first three years of production. Estimates should be on 100% new chemical substance basis. For a Low Volume Exemption application, if you choose to have your notice reviewed at a lower production volume than 10,000 kg/yr, specify the volume and mark (x) in the binding box. If granted, you are bound to this volume

Maximum first 12-month production (kg/yr) (100% new chemical substance basis)	Maximum 12-month production (kg/yr) (100% new chemical substance basis)	Confi-dential	Binding Option Mark (x)
		X	

2. **Use Information** -- You must make separate confidentiality claims for the description of the category of use, the percent of production volume devoted to each category, the formulation of the new substance, and other use information. Mark (X) the "Confidential" Box next to any item you claim as confidential.

a. (1) --Describe each intended category of use of the new chemical substance by function and application. (2) --Mark (X) this column if entry column (1) is confidential business information (CBI). (3) --Indicate your willingness to have the information provided in column (1) binding. (4) --Estimate the percent of total production for the first three years devoted to each category of use. (5) --Mark (X) this column if entry in column (4) is confidential business information (CBI). (6) --Estimate the percent of the new substance as formulated in mixtures, suspensions, emulsions, solutions, or gels as manufactured for commercial purposes at sites under your control associated with each category of use. (7) --Mark (X) this column if entry in column (6) is confidential business information (CBI). (8) --Indicate % of product volume expected for the listed "use" sectors. Mark more than one box if appropriate. Mark (X) to indicate your willingness to have the use type provided in (8) binding. (9) --Mark (X) this column if entry(ies) in column (8) is (are) confidential business information (CBI).

Category of use (1) (by function and application i.e. a dispersive dye for finishing polyester fibers)	CBI (2)	Binding Option Mark (x) (3)	Prod-uction % (4)	CBI (5)	% in Formu lation (6)	CBI (7)	% of substance expected per use (8)					CBI (9)
							Site-limited	Cons-umer	Indus-trial	Com-mercial	Binding Option	
Flame retardant for polyurethane foam			%	X	%	X						X
			%		%							
			%		%							
			%		%							
			%		%							
			%		%							
			%		%							

* If you have identified a "consumer" use, please provide on a continuation sheet a detailed description of the use(s) of this chemical substance in consumer products. In addition include estimates of the concentration of the new chemical substance as expected in consumer products and describe the chemical reactions by which this substance loses its identity in the consumer product.

☐ Mark (X) this box if you attach a continuation sheet.

b. Generic use description — If you claim any category of use description in subsection 2a as confidential, enter a generic description of that category. Read the Instruction Manual for examples of generic use descriptions.

☐ Mark (X) this box if you attach a continuation sheet.

3. **Hazard Information** -- Include in the notice a copy of reasonable facsimile of any hazard warning statement, label, material safety data sheet, or other information which will be provided to any person who is reasonably likely to be exposed to this substance regarding protective equipment or practices for the safe handing, transport, use, or disposal of the new substance. List in part III hazard information you include. — Binding Option Mark (x)

☐ Mark (X) this box if you attach hazard information.

Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE

Section A -- INDUSTRIAL SITES CONTROLLED BY THE SUBMITTER

Mark (X) the "Confidential" box next to any item you claim as confidential

Complete section A for each type of manufacture, processing, or use operation involving the new chemical substance at industrial sites you control. Importers do not have to complete this section for operations outside the U.S.; however, you may still have reporting requirements if there are further industrial processing or use operations after import. You must describe these operations. See instructions manual

1. Operation description

a. Identity -- Enter the identity of the site at which the operation will occur. (Confidential)

Name

Site address (number and street)

City, County, State, ZIP code

If the same operation will occur at more than one site, enter the number of sites. Identify the additional sites on a continuation sheet, and if any of the sites have significantly different production rates or operations, include all the information requested in this section for those sites as attachments. →

☐ Mark (X) this box if you attach a continuation sheet.

b. Type -- Mark (X) ☐ Manufacturing ☐ Processing ☐ Use

c. Amount and Duration -- Complete 1 or 2 as appropriate

1. Batch	Maximum kg/batch (100% new chemical substance)	Hours/batch	Batches/year
2. Continuous	Maximum kg/day (100% new chemical substance)	Hours/day	Days/year

d. Process description ☐ Mark (X) to indicate your willingness to have your process description binding.

(1) Diagram the major unit operation steps and chemical conversions. Include interim storage and transport containers (specify- e.g. 5 gallon pails, 55 gallon drum, rail car, tank truck, etc.).

(2) Provide the identity , the approximate weight (by kg/day or kg/batch on a 100% new chemical substance basis), and entry point of all starting materials and feedstocks (including reactants, solvents, catalysts, etc.), and of all products, recycle streams, and wastes. Include cleaning chemicals (note frequency if not used daily or per batch.).

(3) Identify by number the points of release, including small or intermittent releases, to the environment of the new chemical substance. If releasing to two media at the same step, assign a second release number for the second medium.

--- IMPORT only ---

☐ Mark (X) this box if you attach a continuation sheet.

Confidential

☐

Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE -- Continued

Section A -- INDUSTRIAL SITES CONTROLLED BY THE SUBMITTER -- Continued

2. Occupational Exposure -- You must make separate confidentiality claims for the description of worker activity, physical form of the new chemical substance, number of works exposed, and duration of activity. Mark (X) the "Confidential" box next to any item you claim as confidential.

(1) -- Describe the activities (i.e. bag dumping, tote filling, unloading drums, sampling, cleaning, etc.) in which workers may be exposed to the substance.
(2) -- Mark (X) this column if entry in column (1) is confidential business information (CBI).
(3) -- Describe any protective equipment and engineering controls used to protect workers.
(4) and (6) -- Indicate your willingness to have the information provided in column (3) or (5) binding.
(5) -- Indicate the physical form(s) of the new chemical substance (e.g., solid: crystal, granule, powder, or dust) and % new chemical substance (if part of a mixture) at the time of exposure.
(7) -- Mark (X) this column if entry in column (5) is confidential business information (CBI).
(8) -- Estimate the maximum number of workers involved in each activity for all sites combined.
(9) -- Mark (X) this column if entry in column (8) is confidential business information (CBI).
(10) and (11) -- Estimate the maximum duration of the activity for any worker in hours per day and days per year.
(12) -- Mark (X) this column if entries in columns (10) and (11) are confidential business information (CBI).

Worker activity (i.e., bag dumping, filling drums) (1)	CBI (2)	Protective Equipment/ Engineering Controls (3)	Binding Option Mark (x) (4)	Physical forms(s) and % new substance (5)	Binding Option Mark (x) (6)	CBI (7)	# of Workers Exposed (8)	CBI (9)	Maximum Hrs/day (10)	duration Days/yr (11)	CBI (12)

☐ Mark (X) this box if you attach a continuation sheet.

3. **Environmental Release and Disposal** -- You must make separate confidentiality claims for the release number and the amount of the new chemical substance released and other release and disposal information. Mark (X) the "Confidential" box next to each item you claim as confidential.
   (1) -- Enter the number of each release point identified in the process description, part II, section A, subsection 1d(3).
   (2) -- Estimate the amount of the new substance released (a) directly to the environment or (b) into control technology (in kg/day or kg/batch).
   (3) -- Mark (X) this column if entries in columns (1) and (2) are confidential business information (CBI).
   (4) -- Identify the media (stack air, fugitive air (optional-see Instruction Manual), surface water, on-site or off-site land or incineration, POTW, or other (specify)) to which the new substance will be released from that release point.
   (5) -- a. Describe control technology, if any, and control efficiency that will be used to limit the release of the new substance to the environment. For releases disposed of on land, characterize the disposal method and state whether it is approved for disposal of RCRA hazardous waste. On a continuation sheet, for each site describe any additional disposal methods that will be used and whether the waste is subject to secondary or tertiary on-site treatment. b. Estimate the amount released to the environment after control technology (in kg/day).
   (6) -- Mark (X) this column if entries in columns (4) and (5) are confidential business information (CBI).
   (7) -- Identify the destination(s) of releases to water. Please supply NPDES (National Pollutant Discharge Elimination System) numbers for direct discharges or NPDES numbers of the POTW (Publicly Owned Treatment Works). Mark (X) if the POTW name or NPDES # is confidential business information (CBI).

Release Number (1)	Amount of new substance released (2a)	(2b)	CBI (3)	Medium of release e.g. stack air (4)	Control technology and efficiency (you may wish to optionally attach efficiency data) (5a)	Binding Mark (X)	(5b)	CBI (6)
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐
			☐			☐		☐

(7) Mark (X) the destination(s) of releases to water.		NPDES #	CBI
☐ POTW--provide name(s)			☐
☐ Navigable waterway--provide name(s)			☐
☐ Other--Specify			☐

☐ Mark (X) this box if you attach a continuation sheet.

Part II-- HUMAN EXPOSURE AND ENVIRONMENTAL RELEASE -- Continued

Section B -- INDUSTRIAL SITES CONTROLLED BY OTHERS

Complete section B for typical processing or use operations involving the new chemical substance at sites you do not control. Importers do not have to complete this section for operations outside the U.S.; however, you must report any processing or use activities after import. See the Instructions Manual. *Complete a separate section B for each type of processing, or use operation involving the new chemical substance.* If the same operation is performed at more than one site describe the typical operation common to these sites. Identify additional sites on a continuation sheet.

1(a). Operation Description -- To claim information in this section as confidential, circle or bracket the specific information that you claim as confidential. (1) -- Diagram the major unit operation steps and chemical conversions, including interim storage and transport containers (specify - e.g. 5 gallon pails, 55 gallon drums, rail cars, tank trucks, etc). On the diagram, identify by letter and briefly describe each worker activity. (2) -- Either in the diagram or in the text field 1(b) below, provide the identity, the approximate weight (by kg/day or kg/batch, on an 100% new chemical substance basis), and entry point of all feedstocks (including reactants, solvents and catalysts, etc) and all products, recycle streams, and wastes. Include cleaning chemicals (note frequency if not used daily or per batch). (3) -- Either in the diagram or in the text field 1(b) below, identify by number the points of release, including small or intermittent releases, to the environment of the new chemical substance. (4) Please enter the # of sites (remember to identify the locations of these sites on a continuation sheet): 10 (# of sites)

CBI [X]

Diagram the major unit operation steps and chemical conversions...

1(b). (Optional) This space is for a text description to clarify the diagram above.

[X]

[ ] Mark (X) this box if you attach a continuation sheet.

2. Worker Exposure/Environmental Release

(1) -- From the diagram above, provide the letter for each worker activity. Complete 2-8 for each worker activity described.
(2) -- Estimate the number of workers exposed for all sites combined.
(4) -- Estimate the typical duration of exposure per worker in (a) hours per day and (b) days per year.
(6) -- Describe physical form of exposure and % new chemical substance (if in mixture), and any protective equipment and engineering controls, if any, used to protect workers.
(7) -- Estimate the percent of the new substance as formulated when packaged or used as a final product.
(9) -- From the process diagram above, enter the number of each release point. Complete 9-13 for each release point identified.
(10) -- Estimate the amount of the new substance released (a) directly to the environment or (b) into control technology to the environment (in kg/day or kg/batch).
(12) -- Describe media of release i.e. stack air, fugitive air (optional-see Instructions Manual), surface water, on-site or off-site land or incineration, POTW, or other (specify) and control technology, if any, that will be used to limit the release of the new substance to the environment.
(14) -- Identify byproducts which may result from the operation.
(3), (5), (8), (11), (13) and (15) -- Mark (X) this column if any of the proceeding entries are confidential business information (CBI).

Letter of Activity (1)	# of Workers Exposed (2)	CBI (3)	Duration Of Exposure (4a)	(4b)	CBI (5)	Protective Equip. /Engineering Controls/Physical Form and/ % new substance (6)	% in Formulation (7)	CBI (8)
		X			X			X
		X			X			X
		X			X			X
		X			X			X

Release Number (9)	Amount of New Substance Released (10a)	(10b)	CBI (11)	Media of Release & Control Technology (12)	CBI (13)
			X		X
			X		X
			X		X
			X		X

(14) -- Byproducts: (15) X

☐ Mark (X) this box if you attach a continuation sheet,

OPTIONAL POLLUTION PREVENTION INFORMATION

To claim information in the following section as confidential circle or bracket the specific information that you claim as confidential.

In this section you may provide information not reported elsewhere in this form regarding your efforts to reduce or minimize potential risks associated with activities surrounding manufacturing, processing, use and disposal of the PMN substance. Please include new information pertinent to pollution prevention, including source reduction, recycling activities and safer processes or products available due to the new chemical substance. Source reduction includes the reduction in the amount or toxicity of chemical wastes by technological modification, process and procedure modification, product reformulation, raw materials substitution, and/or inventory control. Recycling refers to the reclamation of useful chemical components from wastes that would otherwise be treated or released as air emissions or water discharges, or land disposal. Descriptions of pollution prevention, source reduction and recycling should emphasize potential risk reduction subsequent to compliance with existing regulatory requirements and can be either quantitative or qualitative. The EPA is interested in the information to assess overall net reductions in toxicity or environmental releases and exposures, not the shifting of risks to other environmental media or non-environmental areas (e.g., occupational or consumer exposure). In addition, information on the relative cost or performance characteristics of the PMN substance to potential alternatives may be provided.

All information provided in this section will be taken into consideration during the review of this substance. See PMN Instructions Manual and Pollution Prevention Guidance manual for guidance and examples.

Optional Pollution Prevention Information (Continued) Describe the expected net benefits, such as (1) an overall reduction in risk to human health or the environment; (2) a reduction in the volume manufactured; (3) a reduction in the generation of waste materials through recycling, source reduction or other means; (4) a reduction in potential toxicity or human exposure and/or environmental release; (5) an increase in product performance, a decrease in the cost of production and/or improved operation efficiency of the new chemical substance in comparison to existing chemical substances used in similar application; or (6) the extent to which the new chemical substance may be a substitute for an existing substance that poses a greater overall risk to human health or the environment.

CBI [X]

☐ Mark (X) this box if you attach a continuation sheet.

Part III -- LIST OF ATTACHMENTS

Attach continuation sheets for sections of the form and test data and other data (including physical/chemical properties and structure/activity information), and optional information after this page. Clearly identify the attachment and the section of the form to which it relates, if appropriate. Number consecutively the pages of any paper attachments. In the column below, enter the inclusive page numbers of each attachment. Electronic attachments can be identified by filename.
Mark (X) the "Confidential" box next to any attachment name you claim as confidential. Read the Instructions Manual for guidance on how to claim any information in an attachment as confidential. You must include with the sanitized copy of the notice form a sanitized version of any attachment in which you claim information as confidential.

#	Attachment name	Attachment Filename	Attachment page number(s)	Confi-dential
1	MSDS: FR-1435 (id# 2037P)	1		☐
2				☒
3				☒
4				☒
5				☒
6				☒
7				☒
				☒
8				☒
9				☒
10				☒
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11				☒
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12				☒
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14				☒
15				☒
16				☒
17				☒
18				☒
19				☒
				☐

☐ Mark (X) this box if you attach a continuation sheet. Enter the attachment name and number.

PHYSICAL AND CHEMICAL PROPERTIES WORKSHEET

To assist EPA's review of physical and chemical properties data, please complete the following worksheet for data you provide and include it in the notice. Identify the property measured, the page of the notice on which the property appears, the value of the property, the units in which the property is measured (as necessary), and whether or not the property is claimed as confidential. If the attachments are electronic, give the attachment number (found on page 12) at (b). The physical state of the neat substance should be provided. These measured properties should be for the neat (100% pure) chemical substance. Properties that are measured for mixtures or formulations should be so noted (% PMN substance in __). You are not required to submit this worksheet; however, EPA strongly recommends that you do so, as it will simplify review and ensure that confidential information is properly protected. You should submit this worksheet as a supplement to your submission of test data. This worksheet is not a substitute for submission of test data.

Property (a)	Mark (X) if provided	Page number (b)	Value (c)	Measured or Estimate (M or E)	Confidential Mark (X) (d)
Physical state of neat substance	☐		☐ (s) ☒ (l) ☐ (g)	M	☐
Vapor pressure @ Temperature 25 °C	☒		7.7x10-7 Pa Torr	M	☐
Density/relative density	☐		2.0 g/cm3	M	☐
Solubility @ Temperature ____ °C Solvent Toluene, Dichloromethane	☐		g/L	M	☐
Solubility in water @ Temperature 20 °C	☒		0.47 mg/L g/L	M	☐
Melting temperature	☐		°C		☐
Boiling / sublimation temperature@____torr pressure	☐		not applicable °C	M	☐
Spectra	☐				☐
Dissociation constant	☐				☐
Particle size distribution	☐				☐
Octanol / water partition coefficient	☒		Log Pow = 5.6	M	☐
Henry's Law constant	☐				☐
Volatilization from water	☐				☐
Volatilization from soil	☐				☐
pH@ concentration ____	☐				☐
Flammability	☐				☐
Explodability	☐				☐
Adsorption / coefficient	☐				☐
Other - Specify Decomposition temperature	☐		> 220C	M	☐
Other - Specify	☐				☐
☐ Mark (X) this box if you attach a continuation sheet. Enter the attachment name and number.					

ATTACHMENT
PAGE 13

- Attachment 1 -

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

1. Identification of the substance & the company

Chemical name The chemical identity of this product is a trade secret.

Chemical formula Proprietary

Chemical family Brominated alkyl aryls

Type of product and use Flame retardant for polymers

Supplier
ICL-IP America Inc.
95 MacCorkle Ave. SW, South Charleston, WV 25303-1411, USA
Tel: (304) 720-3950
Fax: (304) 746-3101

Emergency Telephone Chemtrec (800)424-9300

2. Hazards identification

Emergency overview
Brown viscous liquid
May cause skin sensitization

Potential Health Effects:

- Eye Contact Mild irritant

- Skin contact
Not irritant
May cause sensitization by skin contact.

3. Composition / information on ingredients

Components	CAS No.	Weight %
FR-1435	Confidential	100

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

4. First-aid measures

Eye contact

Holding the eyelids apart, flush eyes promptly with copious flowing water for at least 20 minutes.
Get medical attention immediately.

Skin contact

Remove contaminated clothing. Wash skin thoroughly with mild soap and plenty of water for at least 15 minutes. Wash clothing before re-use.
Get medical attention if irritation occurs.

Inhalation

In case of mist inhalation or breathing fumes released from heated material, remove person to fresh air.
Keep him quiet and warm. Apply artificial respiration if necessary and get medical attention immediately.

Ingestion

If swallowed, wash mouth thoroughly with plenty of water. Get medical attention immediately.
****
NOTE: Never give an unconscious person anything to drink.
****

Notes to the physician

Treat symptomatically and supportively. No specific antidote.

5. Fire - fighting measures

Suitable extinguishing media

Carbon dioxide, dry chemicals, foam, water spray (fog).

Fire fighting procedure

In closed stores, provide fire-fighters with self-contained breathing apparatus in positive pressure mode

Unusual fire and explosion hazards

When heated to decomposition, may release poisonous and corrosive fumes of CO, CO2 and HBr.

6. Accidental release measures

Personal precautions

Wear respirator, chemical safety goggles, rubber gloves and boots

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

Methods for cleaning up Sweep up and shovel into suitable containers for disposal. Avoid raising dust. Ventilate area and wash spill site after material pickup is complete.

7. Handling and storage

Handling Keep containers tightly closed.
Avoid bodily contact.

Storage Store in a dry, cool, well-ventilated area away from incompatible materials (see "materials to avoid").

8. Exposure controls / personal protection

Exposure Limits :

Components	ACGIH-TLV Data	OSHA (PEL) Data
FR-1435	Not determined	Not determined

Ventilation requirements Provide adequate ventilation.

Personal protective equipment:

- **Respiratory protection** Approved respirator
- **Hand protection** Protective gloves
- **Eye protection** Chemical safety goggles
- **Skin and body protection** Body covering clothes and boots

Hygiene measures Do not eat, smoke or drink where material is handled, processed or stored. Wash hands carefully before eating or smoking.

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

9. Physical and chemical properties

Appearance	Brown viscous liquid
Melting point/range	Not applicable
Boiling point/range	Not applicable
Flash point	Not available
Vapour pressure	7.7x10(-7)Pa (25°C)
Vapor density	Not applicable under standard conditions
Solubility:	
- Solubility in water	0.47 mg/l at 20°C
- Solubility in other solvents	Toluene Dichloromethane
Density	2 g/cm³ (25°C)
Decomposition temperature	Above 220°C
Partition coefficient (n-octanol/water)	Log Pow = 5.6

10. Stability and reactivity

Stability	Stable under normal conditions
Materials to avoid	Oxidising agents
Conditions to avoid	Heating above decomposition temperature
Hazardous decomposition products	Hydrogen bromide, carbon dioxide and carbon monoxide
Hazardous polymerization	Not likely to occur

11. Toxicological information

Acute toxicity:	
- Rat oral LD50	>2000 mg/kg
- Rat dermal LD50	>2000 mg/kg
- Eye irritation (rabbit)	Mild irritant
- Dermal irritation (rabbit)	Not irritant
Dermal sensitization	Sensitizer
Chronic toxicity	Not available
Mutagenicity	Not mutagenic by the Ames Test
Carcinogenicity	Not classified by IARC Not included in NTP 11th Report on Carcinogens

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

12. Ecological information

No effects on aquatic organisms are expected to occur at concentrations up to the substances water solubility.

Biodegradation Not readily biodegradable

Bioaccumulative potential Based on BCF results (>800) and the DT50 value for depuration (1.8 days), FR-1435 appears to be only slightly accumulating in fish combined with a highly efficient excretion by the fish. There is no substantial risk for bioconcentration of FR-1435 in fish.

13. Disposal considerations

Waste disposal Observe all federal, state and local environmental regulations when disposing of this material

14. Transportation information

DOT Not regulated
IMO Not regulated

ICAO/IATA Not regulated

15. Regulatory information

USA Not reported in the EPA TSCA Inventory
For Research & Development Use Only

EU Not reported in EINECS
For Research & Development Use Only

16. Other information

This data sheet contains changes from the previous version in section(s)
Product name

ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

This chemical is in early research stages. Therefore, information regarding its properties is still scarce.

Health, Safety & Environment Policy
We will strive to ensure that our operations and products meet the needs of the present global community without compromising the ability of future generations to meet their needs
We accept that the success of our business is dependent on the supply of products and services that will benefit society whilst ensuring human safety and protection of the environment and natural resources
Within the framework of our commitment to the Responsible Care program, we will provide a healthy and safe work environment for employees and will responsibly manage our products at all stages of their life cycle in order to protect human health and the environment whilst maintaining high production standards of operation

TO MEET THIS COMMITMENT WE WILL:
Comply with or exceed applicable national and international regulatory requirements and other requirements to which we subscribe
Communicate openly and actively encourage dialogue with employees, customers and community concerning our products and operations
Implement documented management systems consistent with and for promotion of the Responsible Care ethics
Develop and supply products that can be manufactured, transported, used and disposed of safely whilst best meeting the needs of our customers
Regularly assess, continually improve and responsibly manage health, safety and environmental risks associated with products and processes throughout their life-cycles
Share knowledge and expertise with others and seek to learn from and incorporate improved practices into our own operations
Educate and train employees, contractors and customers to improve their HSE performance
Communicate up-to-date information to enable our workers, customers and other interested parties to handle our products in a safe and environmentally responsible manner
Endeavor to work with customers, suppliers, distributors and contractors to foster the safe use, transport and disposal of our chemicals
Support Product Stewardship programs in cooperation with customers, distributors and transporters

Although the information and recommendations set forth herein (hereinafter "information") are presented in good faith and believed to be correct as of the date hereof, Bromine Compounds Ltd. makes no representations as to the completeness or accuracy thereof.
Information is supplied upon the condition that the persons receiving same will make their own determination as to its safety and suitability for their purposes prior to use.
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ICL Industrial PRODUCTS

MATERIAL SAFETY DATA SHEET

Product Name	**FR-1435**	
Product id	2037P	
Revision date	04/12/2008	**Revision:** 6
Supersedes	09/11/2008	

In an event of discrepancy between the contents of this MSDS and the English version of it, the English version shall prevail.

Prepared By

HSE Division in ISRAEL
telephone: +/972-8-6297830
telefax: +/972-8-6297832
www.icl-ip.com
e-mail:msdsinfo@icl-ip.com

End of safety data sheet

- Attachment 2 -

ICL Industrial
PRODUCTS

BROMINE COMPOUNDS LTD. - RD Division
P.O.Box 180 Beer-Sheva 84101 Israel | www.icl-industrial.com
Dr. N. Komberg
Tel:+972-8-6297017 | Fax:+972-8-6297 534 | kornbergn @icl-ip.com

September 17, 2008

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DAVID DIREKTOR,
IMI TAMI INSTITUTE FOR RESEARCH & DEVELOPMENT LTD.,
INFORMATION AND MARKETING DEPARTMENT
P. O. BOX 10140, HAIFA BAY, 26111, ISRAEL
Tel: +972-4-8469581 Fax: +972-4-8469472
E-mail: library@tami-imi.icl-ip.com

SEARCH PERFORMED FOR
PROJECT NUMBER

CONFIDENTIAL

ON-LINE SEARCH COVER SHEET

- Attachment 4 -

SafePharm Laboratories

FR-1435X:

DETERMINATION OF VAPOUR PRESSURE

DATA REQUIREMENTS:

COUNCIL DIRECTIVE 67/548/EEC, ANNEX V, PART A,
AS PUBLISHED IN COMMISSION DIRECTIVE 92/69/EEC
OECD GUIDELINES FOR TESTING OF CHEMICALS
US EPA OPPTS GUIDELINES

SPL PROJECT NUMBER: 0466/0282

AUTHOR: S P Tremain

STUDY COMPLETION DATE: 26 June 2007

STUDY SPONSOR:

Bromine Compounds Ltd.
Makleff House
P.O.B. 180
BEER-SHEVA 84101
ISRAEL

TEST FACILITY:

Safepharm Laboratories Limited
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

QUALITY ASSURANCE REPORT

This study type is classed as short-term. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

§ 13 April 2007	Protocol Compliance Audit
14 May 2007	Vapour Pressure
§ 06 June 2007	Draft Report Audit
§ Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 27 JUN 2007

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA ACQI FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: 26 JUN 2007

S P Tremain
STUDY DIRECTOR

CONTENTS

FR-1435X:

DETERMINATION OF VAPOUR PRESSURE

SUMMARY

Method. The test procedure was Method A4 specified in Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC) in accordance with Method 104 specified in the OECD Guidelines for Testing of Chemicals, 25 March 2006 and the US EPA Office of Prevention, Pesticides and Toxic Substances Test Guideline 830.7950.

Result. The test material has been determined to have a vapour pressure of 7.7×10^{-7} Pa at 25°C, using a vapour pressure balance.

FR-1435X:

DETERMINATION OF VAPOUR PRESSURE

1. INTRODUCTION

The vapour pressure of the test material has been determined. The method employed was Method A4 specified in Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC), in accordance with Method 104 specified in the OECD Guidelines for Testing of Chemicals, 25 March 2006 and the US EPA Office of Prevention, Pesticides and Toxic Substances Test Guideline 830.7950.

Study Initiation date: 13 April 2007.

Testing was conducted between 10 May 2007 and 13 May 2007.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	FR-1435X
Description	:	pale amber viscous liquid
Batch number	:	38312-2-7
Date received	:	27 April 2007
Storage conditions	:	room temperature in the dark.

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor. The certificate of analysis supplied by the Sponsor is given in Appendix 2.

3. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

4. VAPOUR PRESSURE

4.1 Method

The vapour pressure was determined using a vapour pressure balance with measurements being made at several temperatures and linear regression analysis used to calculate the vapour pressure at 25 °C. Testing was conducted using Method A4 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC), in accordance with Method 104 specified in the OECD Guidelines for Testing of Chemicals, 25 March 2006 and the US EPA Office of Prevention, Pesticides and Toxic Substances Test Guidelines 830.7950.

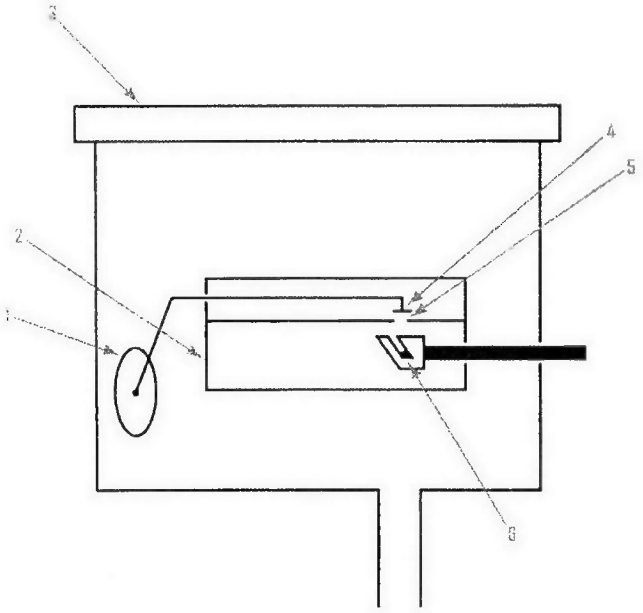
4.1.1 Procedure

The vapour pressure was determined using a vapour pressure balance. The temperature of the sample was controlled electronically. The mass and temperature readings were recorded automatically into a computer file.

A diagram of the cross-section of the vapour pressure balance is represented in Figure 4.1. After evacuating the system, opening the shutter above the sample oven causes the escaping vapour jet to be directed at the scale pan. The difference in mass readings with the orifice covered and uncovered is proportional to the vapour pressure at the given oven temperature.

A sequence of runs was started after a sample of test material had been under vacuum for approximately 18¾ hours. Temperature and pressure readings were taken between 120 and 130°C with a one hour dwell at 120°C between runs.

Figure 4.1 Schematic Diagram of the Apparatus Used



1	Microbalance
2	Oven
3	Glass viewing panel
4	Balance pan
5	Shutter with orifice
6	Test sample

4.1.2 Calculation

The vapour pressure is related to the observed mass difference by Equation 4.1.

Equation 4.1

$$Vp = \frac{\delta m.g}{A}$$

where:

Vp	=	vapour pressure (Pa)
δm	=	mass difference (kg)
g	=	acceleration due to gravity (9.813 m s^{-2})
A	=	area of the orifice ($7.06858 \times 10^{-6} m^2$)

Vapour pressure is related to temperature by Equation 4.2.

Equation 4.2

$$\text{Log}_{10}\,[\text{Vp (Pa)}] = \frac{\text{slope}}{\text{temperature (K)}} + \text{intercept}$$

A plot of Log_{10} Vp (Pa) versus reciprocal temperature (1/T(K)) therefore gives a straight line graph.

The vapour pressure of the sample was measured over a range of temperatures to enable extrapolation to 298.15 K.

4.2 Results

Run 1

Temperature (°C)	Temperature (K)	Reciprocal Temperature (K^{-1})	Mass Difference (µg)	Mass Difference (kg)	Vapour Pressure (Pa)	Log_{10} Vp
123	396.15	0.002524296	44.02	4.402E-08	0.061111038	-1.213880341
124	397.15	0.002517940	47.03	4.703E-08	0.065289689	-1.185155400
125	398.15	0.002511616	53.29	5.329E-08	0.073980173	-1.130884658
126	399.15	0.002505324	61.51	6.151E-08	0.085391639	-1.068584652
127	400.15	0.002499063	65.50	6.550E-08	0.090930781	-1.041289079
128	401.15	0.002492833	67.21	6.721E-08	0.093304699	-1.030096483
129	402.15	0.002486634	75.59	7.559E-08	0.104938286	-0.979066033
130	403.15	0.002480466	79.74	7.974E-08	0.110699549	-0.955854147

A plot of Log_{10} (vapour pressure (Pa)) versus reciprocal temperature (1/T(K)) for Run 1 gives the following statistical data using an unweighted least squares treatment.

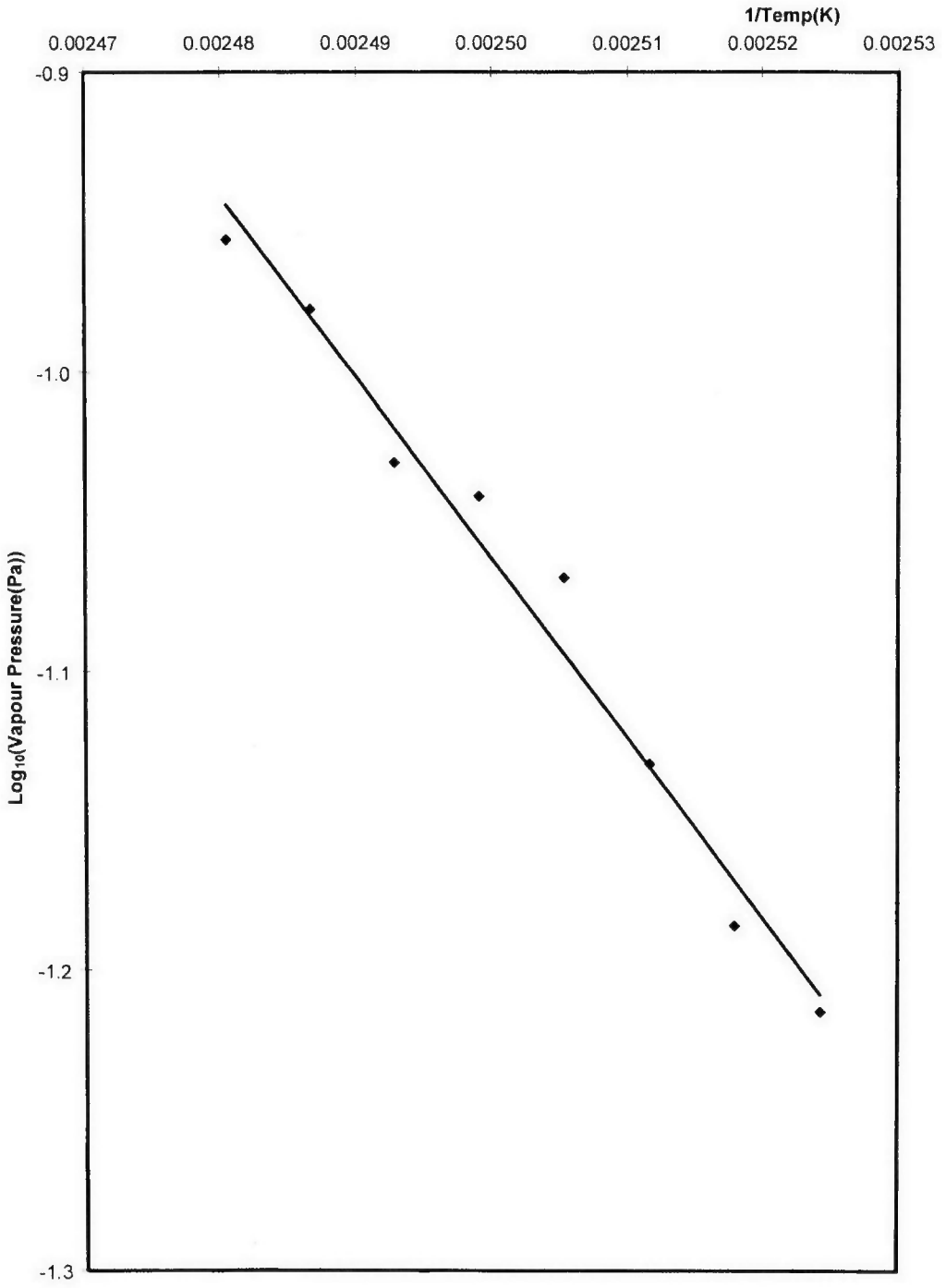
Slope	-6023.025
Standard deviation in slope	376.610
Intercept	13.996
Standard deviation in intercept	0.942

The results obtained indicate the following vapour pressure relationship:

Log_{10} (Vp (Pa)) = -6023.025/temp(K) + 13.996.

The above yields a vapour pressure (Pa) at 298.15 K with a common logarithm of -6.206.

Run 1 - Graph of Log_{10} Vapour Pressure vs Reciprocal Temperature



Run 2

Temperature (°C)	Temperature (K)	Reciprocal Temperature (K^{-1})	Mass Difference (µg)	Mass Difference (kg)	Vapour Pressure (Pa)	Log_{10} Vp
122	395.15	0.002530685	42.31	4.231E-08	0.058737120	-1.231087353
123	396.15	0.002524296	47.03	4.703E-08	0.065289689	-1.185155400
124	397.15	0.002517940	46.38	4.638E-08	0.064387322	-1.191199634
125	398.15	0.002511616	52.56	5.256E-08	0.072966746	-1.136875022
126	399.15	0.002505324	57.77	5.777E-08	0.080199561	-1.095828011
127	400.15	0.002499063	63.14	6.314E-08	0.087654496	-1.057225801
128	401.15	0.002492833	68.75	6.875E-08	0.095442614	-1.020257676
129	402.15	0.002486634	77.38	7.738E-08	0.107423265	-0.968901653
130	403.15	0.002480466	80.55	8.055E-08	0.111824037	-0.951464834

A plot of Log_{10} (vapour pressure (Pa)) versus reciprocal temperature (1/T(K)) for Run 2 gives the following statistical data using an unweighted least squares treatment.

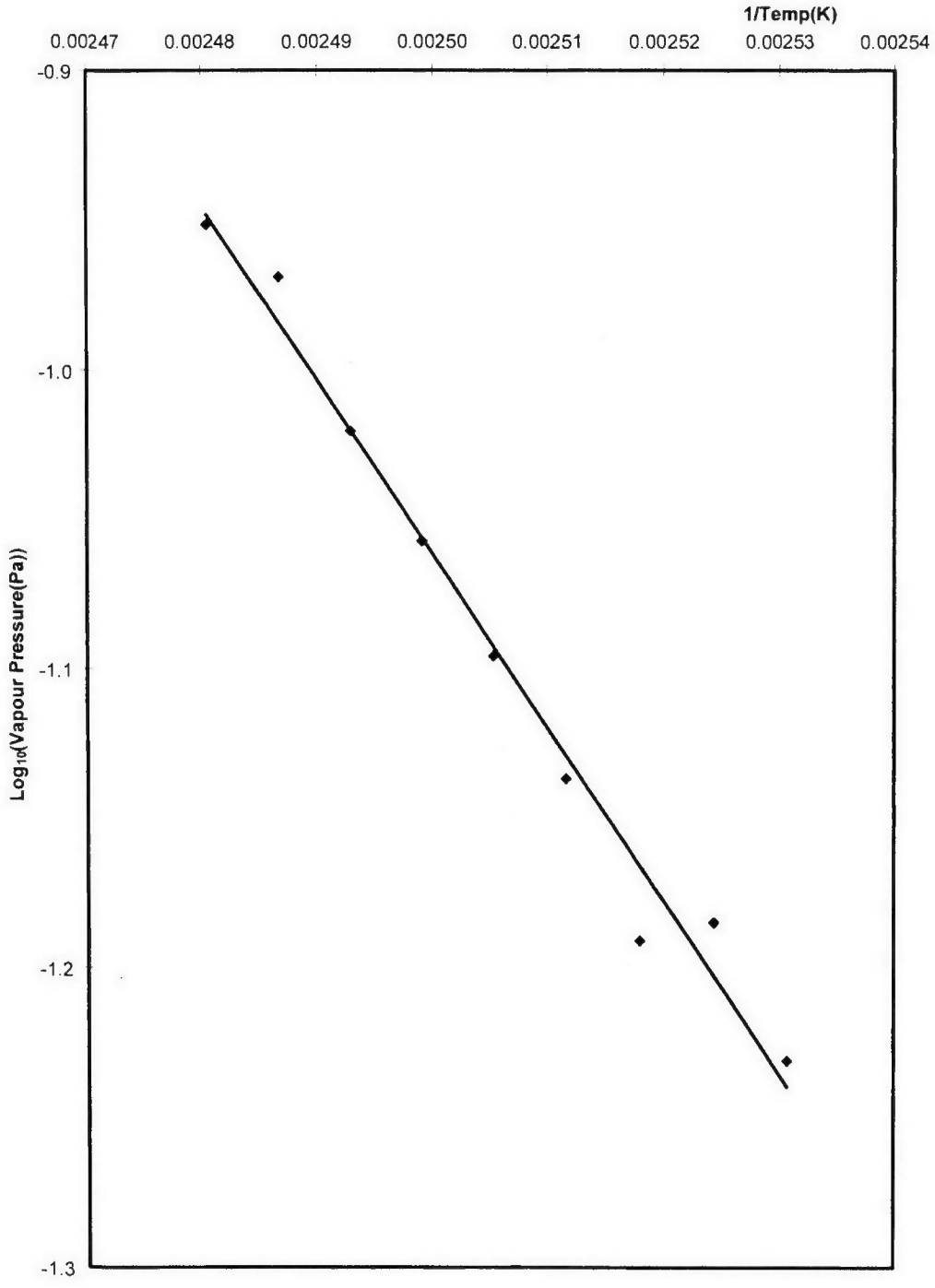
Slope	-5809.758
Standard deviation in slope	284.655
Intercept	13.463
Standard deviation in intercept	0.713

The results obtained indicate the following vapour pressure relationship:

Log_{10} (Vp (Pa)) = -5809.758/temp(K) + 13.463.

The above yields a vapour pressure (Pa) at 298.15 K with a common logarithm of -6.023.

Run 2 - Graph of Log_{10} Vapour Pressure vs Reciprocal Temperature



Run 3

Temperature (°C)	Temperature (K)	Reciprocal Temperature (K^{-1})	Mass Difference (µg)	Mass Difference (kg)	Vapour Pressure (Pa)	Log_{10} Vp
121	394.15	0.002537105	38.32	3.832E-08	0.053197978	-1.274104878
122	395.15	0.002530685	39.46	3.946E-08	0.054780590	-1.261373298
123	396.15	0.002524296	44.59	4.459E-08	0.061902344	-1.208292906
124	397.15	0.002517940	48.90	4.890E-08	0.067885728	-1.168221520
125	398.15	0.002511616	54.84	5.484E-08	0.076131970	-1.118432933
126	399.15	0.002505324	60.54	6.054E-08	0.084045030	-1.075487962
127	400.15	0.002499063	65.17	6.517E-08	0.090472656	-1.043482658
128	401.15	0.002492833	65.74	6.574E-08	0.091263962	-1.039700679
129	402.15	0.002486634	71.28	7.128E-08	0.098954902	-1.004562688
130	403.15	0.002480466	82.75	8.275E-08	0.114878200	-0.939762376

A plot of Log_{10} (vapour pressure (Pa)) versus reciprocal temperature (1/T(K)) for Run 3 gives the following statistical data using an unweighted least squares treatment.

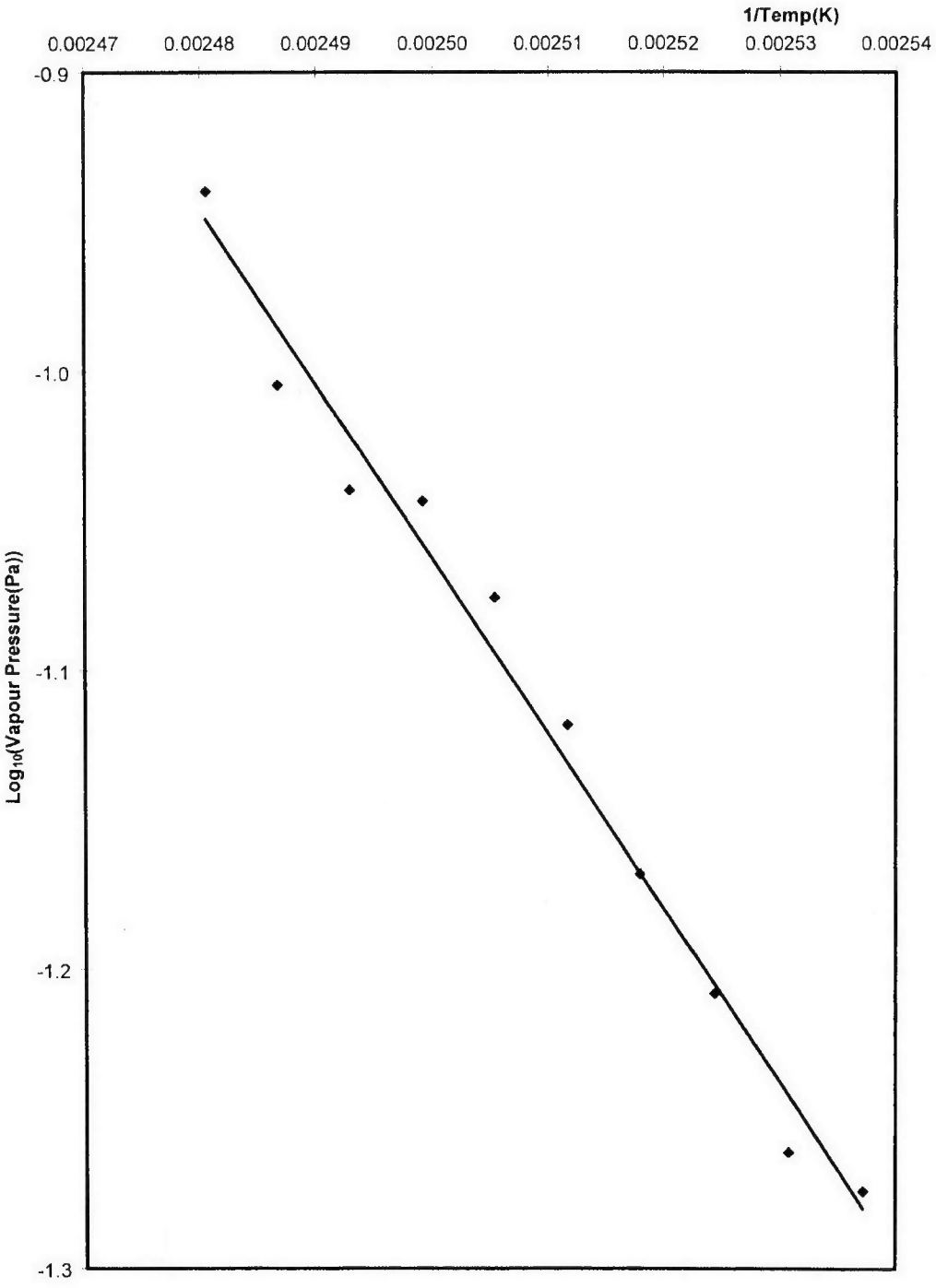
Slope	-5844.128
Standard deviation in slope	271.083
Intercept	13.547
Standard deviation in intercept	0.680

The results obtained indicate the following vapour pressure relationship:

Log_{10} (Vp (Pa)) = -5844.128/temp(K) + 13.547.

The above yields a vapour pressure (Pa) at 298.15 K with a common logarithm of -6.054.

Run 3 - Graph of Log_{10} Vapour Pressure vs Reciprocal Temperature



Run 4

Temperature (°C)	Temperature (K)	Reciprocal Temperature (K^{-1})	Mass Difference (µg)	Mass Difference (kg)	Vapour Pressure (Pa)	Log_{10} Vp
121	394.15	0.002537105	36.45	3.645E-08	0.050601938	-1.295832846
122	395.15	0.002530685	42.39	4.239E-08	0.058848180	-1.230266962
123	396.15	0.002524296	43.37	4.337E-08	0.060208671	-1.220340957
124	397.15	0.002517940	49.96	4.996E-08	0.069357280	-1.158907949
125	398.15	0.002511616	55.57	5.557E-08	0.077145397	-1.112689982
126	399.15	0.002505324	60.45	6.045E-08	0.083920087	-1.076134074
127	400.15	0.002499063	61.67	6.167E-08	0.085613760	-1.067456430
128	401.15	0.002492833	72.09	7.209E-08	0.100079389	-0.999655353
129	402.15	0.002486634	73.55	7.355E-08	0.102106243	-0.990947702
130	403.15	0.002480466	81.36	8.136E-08	0.112948524	-0.947119439

A plot of Log_{10} (vapour pressure (Pa)) versus reciprocal temperature (1/T(K)) for Run 4 gives the following statistical data using an unweighted least squares treatment.

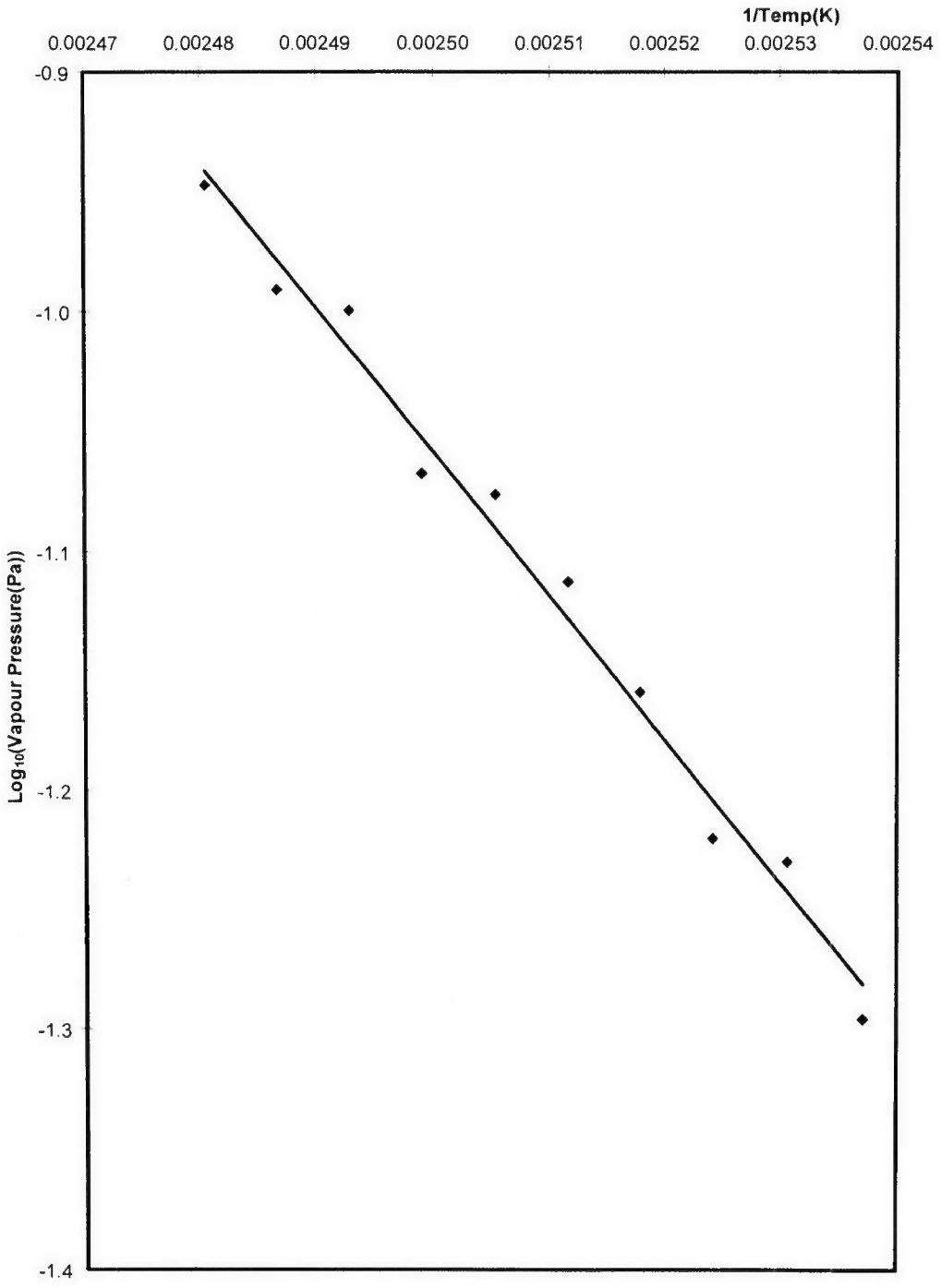
Slope	-6000.153
Standard deviation in slope	261.037
Intercept	13.942
Standard deviation in intercept	0.655

The results obtained indicate the following vapour pressure relationship:

Log_{10} (Vp (Pa)) = -6000.153/temp(K) + 13.942.

The above yields a vapour pressure (Pa) at 298.15 K with a common logarithm of -6.183.

Run 4 - Graph of Log_{10} Vapour Pressure vs Reciprocal Temperature



Summary of Results

Run	Log_{10} [Vp(25°C)]
1	-6.206
2	-6.023
3	-6.054
4	-6.183
Mean	-6.116
Vapour Pressure	7.656×10^{-7} Pa

The test material did not change in appearance under the conditions used in the determination.

4.3 Conclusion

The vapour pressure of the test material has been determined to be 7.7×10^{-7} Pa at 25°C.

Appendix 1 Protocol

SafePharm Laboratories

PROTOCOL

TEST MATERIAL	:	FR-1435X
STUDY TYPE	:	Determination of Vapour Pressure
PROJECT NUMBER	:	0466/0282
PROPOSED START DATE	:	April 2007
PROPOSED COMPLETION DATE	:	May 2007
TARGET (DRAFT) REPORT DATE	:	Mid May 2007
SPONSOR	:	Bromine Compounds Ltd. Makleff House P.O.B. 180 BEER-SHEVA 84101 ISRAEL

APPROVED FOR SPONSOR BY: Ort Menus DATE: 1-4-07

AUTHORISED BY: DATE: 13 APR 2007

S P Tremain
STUDY DIRECTOR

This protocol is issued without signature by the Study Director to enable changes to be made if necessary prior to authorisation. Sponsors should sign and return the document to indicate approval and GLP authorisation will be confirmed by the Study Director's signature prior to the start of the study.

Appendix 1 continued Protocol

DETERMINATION OF VAPOUR PRESSURE

1. INTRODUCTION AND OBJECTIVES

The objective of this study is to determine the vapour pressure of the test material in accordance with the following:

i) Council Directive 67/548/EEC, Annex V, Part A: Methods for the determination of physico-chemical properties, as published in Commission Directive 92/69/EEC.

ii) US EPA, Office of Prevention, Pesticides and Toxic Substances, Series 830: Product Properties Test Guidelines.

iii) OECD Guidelines for Testing of Chemicals.

The work described will be performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

GLP standards are not appropriate to calculated or predicted values/results; therefore the claim of compliance in the final report will exclude any such procedures.

2. TEST FACILITY

Safepharm Laboratories Ltd.
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD

Appendix 1 continued Protocol

3. TEST MATERIAL

Identification

Supplied by the Sponsor with the details of hazardous properties if known. Data relating to the identification, purity and stability of the test material will be the responsibility of the Sponsor.

Storage

Room temperature in the dark unless otherwise specified by the Sponsor.

4. TESTING

Test	Reference
Vapour Pressure	EEC A4, OPPTS 830.7950: OECD 104, 25 March 2006

Appropriate specific test procedures will be documented and approved in the study data.

5. PRINCIPLES OF THE METHOD

The vapour pressure at 25°C will be determined using one of the following:

i) The vapour pressure balance method.

ii) The isoteniscope method.

If the methods are not applicable due to the nature of the test material, the vapour pressure will be calculated using the method detailed in EEC A4 and OECD guideline 104.

6. STUDY RECORDS AND EVALUATION OF DATA

All observations which may be used to interpret the results of the test will be documented.

Where appropriate, mean values and standard deviation will be calculated, and linear regression analysis (least squares method) will be applied.

Appendix 1 continued Protocol

7. QUALITY ASSURANCE

This protocol will be reviewed for GLP compliance and the final report will be audited by Safepharm Quality Assurance Unit. The routine inspection of studies consisting of standard tests is process-based as defined in Quality Assurance Standard Operating Procedures.

8. PROTOCOL AMENDMENTS

Amendments to this protocol will be made only by completion of an Amendment to Protocol form authorised by the Study Director.

9. REPORT

A full report containing a description of the test material, detailed description of the experimental procedures, summary of the observations, discussion and interpretation of the results will be presented. A draft report will be sent to the Sponsor for review and comments before issue of the final report.

The report will be presented in a format that is acceptable to the US EPA.

10. ARCHIVE

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years after which instructions will be sought as to further retention or disposal. Further retention or return of the data will be chargeable to the Sponsor.

Appendix 2 Certificate of Analysis

ICL Industrial PRODUCTS

IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel:972-4-8469553 Fax:972-4-8469320 zilbermanj@tami-imi.icl-ip.com

April 10, 2007

Certificate of Analysis

CONFIDENTIAL

Batch 38312-2-7	
Appearance, Color	Yellow-amber liquid
Assay, % (GC area)	>98
Water, ppm	<500
Br total, %	63
Density, g/cm^3 at 25°C	~2.0
Viscosity, cps at 23°C	1800-2400
Acidity, meq/g	<0.1
Solubility, g/100 g Water Toluene	 <0.1 complete
Expiration date	10.04.08

Best regards

Dr. Yossi Zilberman

Appendix 3 Statement of GLP Compliance in accordance with Directive 2004/9/EC

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan J Wright 21/11/05.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority

FR-1435X

OCTANOL/WATER PARTITION COEFFICIENT

Sponsor

Dead Sea Bromine Group
P.O. Box 180
Beer Sheva
84101
ISRAEL

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Final: 8 September 2004

CONTENTS

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

FR-1435X

Octanol/Water Partition Coefficient

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8), as amended by EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

The stability of the test substance was the responsibility of the Sponsor.

P. Sydney

8 September 2004

P. Sydney, B.Sc., M.Sc.
Study Director
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

FR-1435X

Octanol/Water Partition Coefficient

The following inspections and audits have been carried out in relation to this study:

Study Phase	**Date(s) of Inspection**	**Date of Reporting to Study Director and Management**
Protocol Audit	26 February 2004	26 February 2004
Study Based Inspections		
Test vessel set up	21 May 2004	21 May 2004
Sampling	24 May 2004	24 May 2004
Report Audit	9-11 June 2004	11 June 2004

In addition, process based inspections were conducted of other routine and repetitive procedures employed on this type of study at or about the time this study was in progress. Similarly an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were reported to Company Management.

H. Comb

8 September 2004

H. Comb, B.Sc., M.R.Q.A.
Group Manager
Department of Quality Assurance
Huntingdon Life Sciences Ltd.

Date

RESPONSIBLE PERSONNEL

FR-1435X

Octanol/Water Partition Coefficient

The following staff member has reviewed this report.

A. L. Comb, B.Sc., Ph.D.
(Scientific Manager, Product Chemistry)

The following staff were responsible for the conduct of the work and reporting of the results.

P. Sydney, B.Sc., M.Sc.
(Study Director, Product Chemistry)

C. Steil, B.Sc.
(Scientist, Product Chemistry)

SUMMARY

A study was performed to determine the octanol/water partition coefficient of FR-1435X.

The method followed is described in a OECD Draft Guideline - Partition Coefficient (Octanol/Water): Slow Stirring Method, November 2003.

FR-1435X was found to have a $\log_{10}P_{ow}$ value of 5.6.

INTRODUCTION

The study was performed to determine the octanol/water partition coefficient of FR-1435X.

The protocol was approved by the Study Director and Huntingdon Life Sciences Management on 24 February 2004, and by the Sponsor on 25 February 2004.

The experimental start and completion dates were 17 March 2004 and 3 June 2004 respectively.

Location of study : Eye Research Centre
Eye
Suffolk
IP23 7PX

Primary data from the tests, and a copy of the final report, are stored in the archives of Huntingdon Life Sciences.

TEST SUBSTANCE

CONFIDENTIAL

Identity:	FR-1435X
Appearance:	Brown liquid
Storage conditions:	Room temperature
Lot number:	38278-53-4
Purity:	97.2%
Date received:	22 December 2003

OCTANOL/WATER PARTITION COEFFICIENT
(OECD Draft Guideline, November 2003)

DEFINITION AND UNITS

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents, in this case, n-octanol and water.

$$P_{ow} = \frac{C_{n\text{-}octanol}}{C_{water}}$$

The partition coefficient is usually given in the form of its logarithm to base ten ($\log_{10}P_{ow}$).

PRELIMINARY ESTIMATION

A value of $\log_{10}P_{ow}$ for FR-1435X was estimated by a calculation method using a LOGKOW computer program (Version 1.63, Syracuse Research Corporation). The program estimates the $\log_{10}P_{ow}$ value by reference to compound structures for which reliable $\log_{10}P_{ow}$ data is available.

CONFIDENTIAL

MAIN TEST

Solvent preparation:

Volumes of n-octanol and purified water were mixed together for a minimum period of 2 days to pre-saturate each phase with respect to the other. The organic and aqueous layers were then transferred to separate storage bottles.

Preparation of n-octanol stock solutions:

Stock solutions of nominal concentration 10 g/l with respect to FR-1435X were prepared in water saturated n-octanol. Dilutions of the stock solution were prepared and analysed before the test to confirm that the test substance had fully dissolved, and that the stock solution was then suitable for use.

Procedure:

A pilot experiment was conducted initially to determine the approximate concentration of FR-1435X in the n-octanol and aqueous phases. Following this, the definitive procedure detailed below was conducted in triplicate:

n-Octanol saturated water (950 ml) was added to the test vessel (Figure 1), and allowed to equilibrate at test temperature (25 ± 0.5°C). Stock solution (50 ml) was then added slowly by pipetting against the wall of the test vessel in order to avoid turbulent mixing of the phases. Slow-stirring was started, the stirring rate being adjusted to produce a vortex depth of between 0.5 and 2.5 cm at the water/n-octanol interface. Stirring was provided by a magnetic stirrer, with a teflon-coated magnetic stir bar (length 45 mm, diameter 8 mm). The vessel was covered with aluminium foil to exclude light.

At intervals, stirring was stopped and the liquids allowed to stop moving before sampling. The respective phases were treated as follows, before slow-stirring was resumed.

An aliquot (100 µl) of the upper n-octanol phase was removed using an all glass-metal syringe and diluted to 50 ml with methanol for analysis by ultraviolet (UV) spectrophotometry.

A sample of the lower aqueous phase (5 ml, measured gravimetrically to avoid unnecessary transfer stages) was diluted to 10 ml with methanol for analysis by high performance liquid chromatography (HPLC). The aqueous phase was sampled from the stop-cock at the bottom of the test vessel after allowing the dead volume of water in the tap to run to waste. An additional portion of the aqueous phase was removed on each sampling occasion for pH measurement.

Sampling continued until the determined $\log_{10}P_{ow}$ value was consistent for at least four timepoints. For Test 2, excessive stirring initially (5 cm vortex) resulted in a lengthened equilibration time.

ANALYTICAL METHODS

UV spectrophotometry conditions:

Instrument:	Unicam 8755 UV-Visible Spectrophotometer
Cell type:	Quartz
Cell path length:	10 mm
Wavelength:	245 nm
Slit width:	2.0 nm

HPLC conditions:

Instrument:	Hewlett Packard 1050 Liquid Chromatograph
Column:	Zorbax RX-C18 (25 cm x 4.6 mm internal diameter)
Column temperature:	Ambient
Mobile phase composition:	Methanol:water (9:1 v/v)
Flow rate:	1.2 ml/min
Injection volume:	100 µl
Detector:	UV set at 225 nm
Retention time:	The test substance chromatographed as two peaks, in a 1:1 ratio, at retention times of approximately 10 and 11 minutes. The summed areas of the peaks were used for quantification purposes.

PREPARATION OF CALIBRATION

A stock calibration solution of concentration 1027 mg/l was prepared by weighing test substance (25.68 mg) into a 25 ml volumetric flask and dissolving in and diluting to volume with methanol.

UV spectrophotometric analysis:

Calibration solutions in the range 5 to 50 mg/l were prepared by dilutions of the stock solution with methanol.

HPLC analysis:

Calibration solutions in the range 0.005 to 0.1 mg/l were prepared by dilutions of the stock solution with methanol:water (1:1 v/v).

CALCULATIONS

n-octanol phases:

The concentration of FR-1435X in the analysed solution (C_A) was calculated by insertion of the determined sample absorbance value into the calibration linear regression equation.

The concentration of FR-1435X in the n-octanol phase (C_B) was then calculated thus:

$$C_B \text{ (mg/l)} = C_A \text{ (mg/l)} \times \text{dilution factor}$$

Aqueous phases:

The concentration of FR-1435X in the analysed solution (C_A) was calculated from standards introduced before and after samples (bracketing standards) by the following equation:

$$C_A \text{ (mg/l)} = \frac{\text{sample peak area x standard concentration (mg/l)}}{\text{mean peak area of bracketing standards}}$$

The concentration of FR-1435X in the aqueous phase (C_B) was then calculated thus:

$$C_B \text{ (mg/l)} = C_A \text{ (mg/l)} \times \text{dilution factor}$$

RESULTS

The calibration for the UV spectrophotometric analysis was found to be linear over the range 0 to 50 mg/l of standard solutions in methanol with a regression coefficient of 1.0000 (Table 1, Figure 2).

The calibration for the HPLC analysis was found to be linear over the range 0 to 0.1 mg/l of standard solutions in methanol:water (1:1 v/v) with a regression coefficient of 0.9996 (Table 2, Figure 3). Further details of method validation for analysis of FR-1435X in n-octanol saturated water can be found in Huntingdon Life Sciences Report No. DSV013/042538.

Table 3 presents a summary of the partition coefficient data from Test 1, and shows a measured $\log_{10}P_{ow}$ value of 5.6.

Tables 4 and 5 present the primary data for the n-octanol and aqueous phases from Test 1 respectively.

Tables 6 to 8, and Tables 9 to 11, show results from Tests 2 and 3 respectively. As in Test 1, a $\log_{10}P_{ow}$ value of 5.6 was obtained in each case, yielding an overall mean value of 5.6.

CONCLUSION

FR-1435X was determined to have a $\log_{10}P_{ow}$ value of 5.6.

FIGURE 1

Test vessel for the slow-stirring method

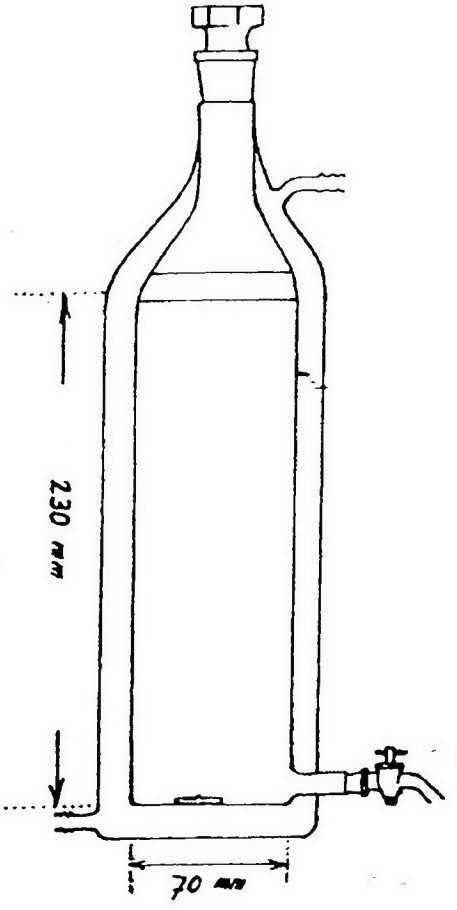


TABLE 1

Standard calibration for FR-1435X by UV spectrophotometry

Standard concentration (mg/l)	Absorbance (245 nm)
51.36	1.307
41.09	1.048
30.82	0.792
20.54	0.527
10.27	0.270
5.136	0.134

Linear regression (including x = 0, y = 0) $y = 0.02542x + 0.00448$, $r = 1.0000$

x = concentration
y = absorbance

FIGURE 2

Standard calibration for FR-1435X by UV spectrophotometry

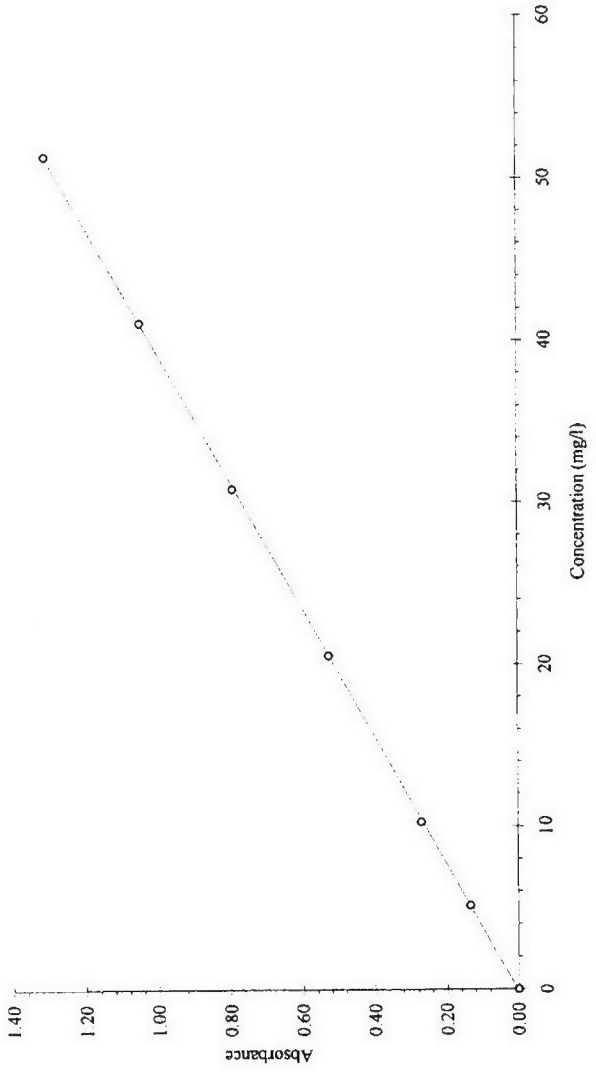


TABLE 2

Standard calibration for FR-1435X by HPLC

Standard concentration (mg/l)	Peak area
0.1027	29.615
0.08218	23.409
0.06163	17.658
0.04109	11.916
0.02054	6.6969
0.01027	2.8456
0.005136	1.3054

Linear regression (including x = 0, y = 0) $y = 286.4x + 0.0977$, $r = 0.9996$

x = concentration
y = peak area

FIGURE 3

Standard calibration for FR-1435X by HPLC

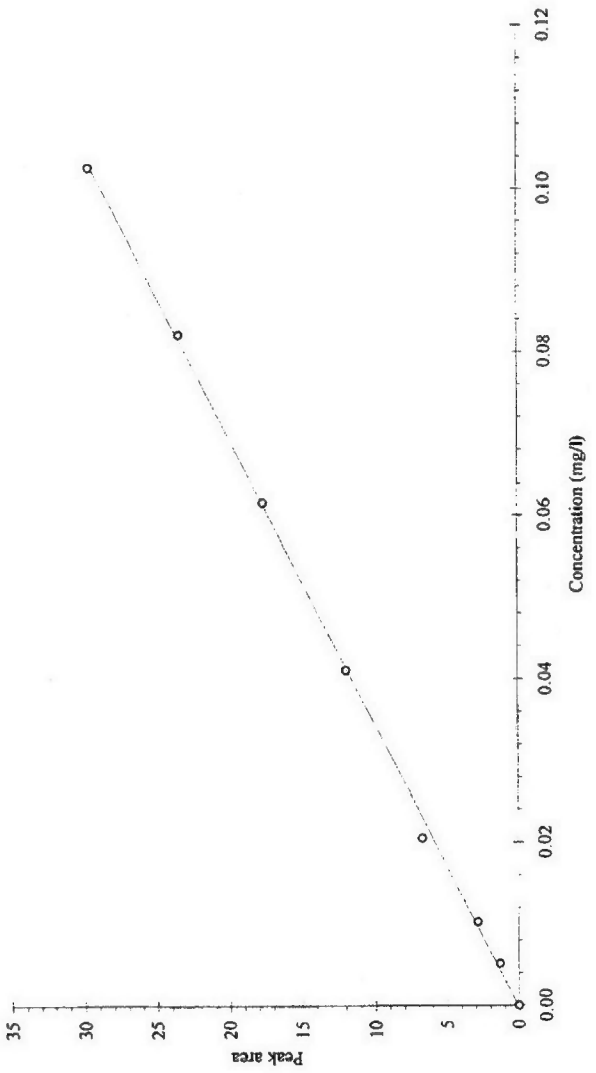


TABLE 3

Summary of partition coefficient data (Test 1)

Sample	Sampling time (hours)	n-octanol phase concentration (mg/l)	Aqueous phase concentration (mg/l)	$Log_{10}P_{ow}$	pH of aqueous phase after partition
A	41	10960	0.02272	5.68	7.96
B	46	10380	0.02877	5.56	7.78
C	64	10670	0.02550	5.62	8.09
D	68	10500	0.02515	5.62	7.94
E	71	10350	0.02072	5.70	8.09

Mean $log_{10}Pow = 5.64 \pm 0.06$

TABLE 4

UV spectrophotometric analysis of n-octanol phases (Test 1)

Sample	Absorbance	C_A (mg/l)	Dilution factor	C_B (mg/l)
n-octanol A	0.541	21.92	500	10960
n-octanol B	0.513	20.76	500	10380
n-octanol C	0.508	21.33	500	10670
n-octanol D	0.500	21.00	500	10500
n-octanol E	0.493	20.71	500	10350

Linear regression equation used to calculate concentrations for samples A and B: $y = 0.02417x + 0.01111$
Linear regression equation used to calculate concentrations for samples C to E: $y = 0.02410x - 0.00601$

where x = concentration, y = absorbance

TABLE 5

HPLC analysis of aqueous phases (Test 1)

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (mg/l)
0.02054 mg/l std	7.5260	-	-	-
Aqueous A	4.1802	0.01136	2	0.02272
Aqueous B	5.2912	0.01438	2	0.02877
0.02054 mg/l std	7.5887	-	-	-
0.02054 mg/l std	8.2150	-	-	-
Aqueous C	4.6644	0.01275	2	0.02550
Aqueous D	4.5999	0.01257	2	0.02515
Aqueous E	3.7904	0.01036	2	0.02072
0.02054 mg/l std	6.8166	-	-	-

TABLE 6

Summary of partition coefficient data (Test 2)

Sample	Sampling time (hours)	n-octanol phase concentration (mg/l)	Aqueous phase concentration (mg/l)	$Log_{10}P_{ow}$	pH of aqueous phase after partition
A	66	10300	0.2417	4.63	7.70
B	71	9829	0.2190	4.65	7.83
C	89	9847	0.1110	4.95	7.64
D	92	9847	0.1083	4.96	7.73
E	96	9827	0.09641	5.01	7.77
F	137	9900	0.05266	5.27	7.80
G	143	9881	0.05788	5.23	7.98
H	161	10010	0.04826	5.32	7.91
I	164	9908	0.04452	5.35	7.93
J	167	9868	0.04252	5.37	7.54
K	261	9987	0.02900	5.54	7.71
L	264	9730	0.02657	5.56	7.85
M	281	9947	0.02807	5.55	7.93
N	284	9868	0.02783	5.55	8.09
O	287	9750	0.02567	5.58	7.82

Mean $log_{10}Pow = 5.56 \pm 0.02$ (excluding samples A to J, as equilibrium not yet established)

TABLE 7

UV spectrophotometric analysis of n-octanol phases K to O (Test 2)

Sample	Absorbance	C_A (mg/l)	Dilution factor	C_B (mg/l)
n-octanol K	0.509	19.97	500	9987
n-octanol L	0.496	19.46	500	9730
n-octanol M	0.507	19.89	500	9947
n-octanol N	0.503	19.74	500	9868
n-octanol O	0.497	19.50	500	9750

Linear regression equation used to calculate concentrations for samples K to O: $y = 0.02531x + 0.00358$

where x = concentration, y = absorbance

TABLE 8

HPLC analysis of aqueous phases K to O (Test 2)

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (mg/l)
0.02054 mg/l std	6.4909	-	-	-
Aqueous K	4.4961	0.01450	2	0.02900
Aqueous L	4.1204	0.01329	2	0.02657
0.02054 mg/l std	6.2507	-	-	-
0.02054 mg/l std	6.2507	-	-	-
Aqueous M	4.4027	0.01403	2	0.02807
Aqueous N	4.3646	0.01391	2	0.02783
Aqueous O	4.0270	0.01284	2	0.02567
0.02054 mg/l std	6.6389	-	-	-

TABLE 9

Summary of partition coefficient data (Test 3)

Sample	Sampling time (hours)	n-octanol phase concentration (mg/l)	Aqueous phase concentration (mg/l)	$Log_{10}P_{ow}$	pH of aqueous phase after partition
A	66	10320	0.02496	5.62	7.85
B	71	10080	0.02779	5.56	7.91
C	89	9965	0.02301	5.64	7.80
D	92	9965	0.02627	5.58	7.69
E	96	10000	0.02203	5.66	7.91

Mean $log_{10}Pow = 5.61 \pm 0.04$

TABLE 10

UV spectrophotometric analysis of n-octanol phases (Test 3)

Sample	Absorbance	C_A (mg/l)	Dilution factor	C_B (mg/l)
n-octanol A	0.526	20.63	500	10320
n-octanol B	0.514	20.17	500	10080
n-octanol C	0.511	19.93	500	9965
n-octanol D	0.511	19.93	500	9965
n-octanol E	0.513	20.01	500	10000

Linear regression equation used to calculate concentrations for samples A and B: $y = 0.02564x - 0.00304$
Linear regression equation used to calculate concentrations for samples C to E: $y = 0.02542x + 0.00448$

where x = concentration, y = absorbance

TABLE 11

HPLC analysis of aqueous phases (Test 3)

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (mg/l)
0.02054 mg/l std	6.0513	-	-	
Aqueous A	3.6760	0.01248	2	0.02496
Aqueous B	4.0936	0.01390	2	0.02779
0.02054 mg/l std	6.0528	-	-	
0.02054 mg/l std	6.8094	-	-	-
Aqueous C	3.8052	0.01150	2	0.02301
Aqueous D	4.3440	0.01313	2	0.02627
Aqueous E	3.6435	0.01101	2	0.02203
0.02054 mg/l std	6.7816	-	-	-

APPENDIX 1

STUDY PROTOCOL

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

CONFIDENTIAL

PROTOCOL

FR-1435X

OCTANOL/WATER PARTITION COEFFICIENT

Sponsor

Dead Sea Bromine Group
P.O. Box 180
Beer Sheva
84101
ISRAEL

Research Laboratory

Huntingdon Life Sciences Limited
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 14

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

CONTACT DETAILS

Sponsor	:	Dr Sarit Lifshitz
Address	:	Dead Sea Bromine Group P.O. Box 180 Beer Sheva 84101 ISRAEL
Telephone No	:	(972)-8-6297672
Fax No	:	(972)-8-6297832
E-Mail:		lifshitzs@dsbg.com

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

PROTOCOL APPROVAL

FR-1435X

OCTANOL/WATER PARTITION COEFFICIENT

P. Sydney

24 February 2004

P. Sydney, B.Sc., M.Sc.
Study Director
Huntingdon Life Sciences Ltd

Date

A. L. Comb

24 February 2004

A. L. Comb, B.Sc., Ph.D.
Management
Huntingdon Life Sciences Ltd

Date

Sarit Lifshitz

25.2.04

Sponsor
Dead Sea Bromine Group

Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

PLEASE NOTE THAT THE STUDY CANNOT BEGIN UNLESS HUNTINGDON LIFE SCIENCES IS IN RECEIPT OF A PROTOCOL APPROVAL SIGNED BY THE SPONSOR

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

FR-1435X

OCTANOL/WATER PARTITION COEFFICIENT

Number of pages for internal distribution: 11

This working document is approved for circulation and use:

P. Sydney

P. Sydney, B.Sc., M.Sc.
Study Director

24 February 2004

Date

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

CONTENTS

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

1. INTRODUCTION

1.1 Objective and reason for study

Information on physicochemical properties is important in the assessment of the potential hazards of a substance during manufacture, transportation and storage, is useful in the characterisation of the material and aids in the prediction if its environmental fate. In addition, knowledge of certain physicochemical properties is useful for performing and interpreting toxicological and ecotoxicological studies.

1.2 Regulatory acceptance

The study is designed in accordance with accepted principles in order to meet the requirements of the OECD Guidelines for the Testing of Chemicals.

1.3 Good Laboratory Practice

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

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Huntingdon Life Sciences

2. STUDY DETAILS

2.1 Study Director

P. Sydney, B.Sc., M.Sc.

In the temporary absence of the Study Director, routine duties and technical queries will be attended to by A. L. Comb; formal GLP responsibilities will be assumed by Management.

2.2 Testing laboratory

Name:	Huntingdon Life Sciences Limited
Address:	Department of Product Chemistry, Eye Research Centre, Eye, Suffolk, IP23 7PX.
Telephone:	+44 (0) 1379 672033
Facsimile:	+44 (0) 1379 672471
E-Mail:	SydneyP@UKOrg.Huntingdon.com

2.3 Study schedule

Study No.:	DSV/012
Experimental start:	April 2004
Completion:	May 2004
Despatch of draft report:	May 2004

The actual dates will be detailed in the final report.

2.4 Health and Safety

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work Act 1974, and the current Control of Substances Hazardous to Health Regulations, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

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Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

2.5 Test substance

CONFIDENTIAL

Identity:	FR-1435X
Appearance:	Brown liquid
Storage conditions:	Room temperature
Lot number:	38278-53-4
Expiry:	To be included in the raw data if/when available
Purity:	97.2%

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3. EXPERIMENTAL PROCEDURE

3.1 Method

The method will be conducted as described in OECD Draft Guideline, Partition Coefficient (Octanol/Water) Slow Stirring Method, November 2003.

3.2 Definition and units

The partition coefficient between water and octanol (P_{ow}), is defined as the ratio of equilibrium concentrations of the test substance in 1-octanol saturated with water (C_o) and water saturated with 1-octanol (C_w):

$$P_{ow} = \frac{C_o}{C_w}$$

Most frequently it is given as the logarithm to the base 10 ($\log P_{ow}$)

3.3 Preliminary estimation

The partition coefficient will be estimated by a calculation method (for example using the computer programme KOWWIN) or by using the ratio of solubilities in octanol and water.

3.4 Main test

3.4.1 Preparation of test solvents

1-Octanol with a purity of least +99% will, if necessary, be purified by extraction with acid, base and water and subsequent drying. Water obtained from a purification system (Elgastat Maxima) will be used in the determination.

Octanol and water will be mutually saturated prior to the experiment by slow stirring the two phases for two days.

3.4.2 Preparation of test solution

An appropriate amount of test substance will be selected and dissolved in 1-octanol (saturated with water) to produce a clear solution devoid of suspended solids. The concentration used will be such that the concentration will not exceed 0.1M in either phase.

If the estimated value of $\log P_{ow}$ exceeds 5 then an octanol/test substance stock solution will be prepared and left until stable concentration values are attained by analysis. The stock solution will then be diluted and analysed for test substance. If the measured concentration is consistent with the dilution then the test solution can be used in the slow stir test.

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3.4.3 Octanol/water volume ratios

The volume of water and octanol will be selected on consideration of the limit of quantitation of the analytical method, extraction concentration factor, volumes sampled and expected concentrations in the test phases. Typical phase ratios used for the determination of compounds with log P of 4.5 or higher are 20 to 50 ml 1-octanol and 950 to 980 ml water. Whatever phase ratio is decided upon, the upper 1-octanol layer must be at least 0.5 cm thick to allow for sampling of the phase without disturbing it.

3.4.4 Determination of the equilibration time

A pilot experiment will be undertaken to determine the concentration of test substance in the octanol and water phases. According to the test method the pilot study should also be used to determine the equilibration time for stirring, however since this also forms part of the main test it is considered not to be necessary in the pilot study.

3.4.5 Analytical method validation

Validation of the analytical method for the aqueous phase will be undertaken and reported in a separate study.

3.4.6 Test conditions

The test will be performed at 25°C ± 0.5°C.

The test vessel will be protected from light by either performing the experiment in a dark room or by covering the test vessel with aluminium foil.

The test method requires the partition coefficient of ionisable substances to be determined in both their ionised and unionised states by the use of suitable (non-complexing) buffer solutions. However, as FR1435X contains no functional groups that would readily ionise the partition coefficient will be determined with ultra- pure water only.

3.4.7 Performance of the test

1-Octanol-saturated water will be added to the test vessel and allowed to equilibrate to the test temperature. The desired amount of test substance will be dissolved in the required volume of 1-octanol saturated with water and the solution will then be carefully added to the test vessel. In order to avoid turbulent mixing of the phases the 1-octanol phase will be pipetted slowly against the wall of the test vessel and just above the surface of the water phase. Decantation of 1-octanol directly into the flask will be avoided and drops of 1-octanol will not be allowed to fall directly into the water.

Stirring will be started and slowly increased until a vortex at the water/1-octanol interface is created with a depth between 0.5 and 2.5cm. The stirring rate will be reduced if the vortex depth of 2.5 cm is exceeded, otherwise microdroplets of 1-octanol may be formed, leading to an overestimation of the test substance concentration in the water. Stirring will be stopped prior to sampling and the liquid phases allowed to stop moving.

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1-Octanol samples will be obtained by withdrawing a small aliquot (ca 100µl) from the 1-octanol layer with a 100 microliter all glass-metal syringe. Care will be taken not to disturb the phase interface. The volume of the sample taken will be recorded.

The water phase will then be sampled from the stop-cock at the bottom of the test vessel after allowing the dead volume of water in the tap to go to waste (the water in the tap is not stirred and therefore not in equilibrium with the bulk). The water sample will be collected from the tap directly into a separating funnel containing the extraction solvent. The flow will be such that the water/1-octanol layer in the test vessel is not disturbed. In order to avoid unnecessary sample transfer steps the sample volume will be determined gravimetrically.

3.4.8 Extraction and analysis of samples

Analytical recoveries of the test substance from the water phase and the 1-octanol phase will be established prior to the experiment.

The octanol phase will be analysed either directly or after dilution with a solvent compatible with the method of analysis to be used. A surrogate standard may be used if the recovery experiments demonstrate a high degree of variation (>10%).

The water phase will be extracted with a suitable high purity solvent (residue analysis grade). The extract solvent will be removed by rotary film evaporator or nitrogen blowdown, and the residue re-dissolved in a solvent compatible with the method of analysis to be used. If the preliminary estimation of log Pow is greater than 6 then a surrogate standard will be spiked to the water sample prior to extraction in order to correct for losses occurring during extraction and concentration of the water samples.

Three slow-stirring experiments will be performed under identical conditions and samples will be taken until four successive time points yields a slope that is not significantly different from 0 at a p-level of 0.05 indicating that log C_o/C_w is independent of time.

3.5 Treatment of results

The value of log P_{ow} for each experiment will be calculated as the weighted average value of log C_o/C_w for the part of the curve C_o/C_w versus time, for which equilibrium has been demonstrated.

The average value of log P_{ow} for different experiments will be calculated as the average of the results of the individual experiments weighted with their respective variances.

The error of the average of log P_{ow} will be estimated as the repeatability of log C_o/C_w determined during the equilibrium phase in the individual experiments.

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4. REPORTING

The results of these studies will be reported to the Sponsor to include, but not necessarily be limited to, the following:

Test substance:

common name, chemical name, CAS number, Structural formula, and relevant physicochemical properties (where available).

purity (impurities).

the preliminary estimate of log Pow and the method used to derive the value.

Test conditions:

dates of performance of the study.

temperature during the experiment.

volumes of 1-octanol and water at the beginning of the test.

volumes of 1-octanol and water withdrawn.

volumes of 1-octanol and water remaining in the test vessels.

description of the test vessels and stirring conditions including geometry of the stirring bar and test vessel, vortex height in mm and, if available, stirring rate.

analytical methods used to determine the test substance and the method limit of quantitation.

sampling times.

number of replicates.

Results:

repeatability and sensitivity of the analytical methods used.

determined concentrations of the test substance in 1-octanol and water as a function of time.

demonstration of mass balance.

temperature or range of temperature during the experiment.

the regression of concentration ratio against time.

the average value log $P_{ow,Av}$ and its standard error.

discussion and interpretation of the results.

examples of raw data figures of representative analysis, including recoveries of surrogates and the number of levels used in the calibration and the correlation coefficient of the calibration curve.

results of QA/QC.

when available: validation report of the assay procedure (to be indicated among references).

Two types of report are issued:

Draft report:	After QA audit, for review by the Sponsor
Final report:	After approval by the Sponsor

Routinely, reports are supplied on A4 paper and the following number are supplied:

Draft report:	1 unbound
Final report	1 bound (with original signature pages) and 1 unbound

Any additions or corrections to an authorised final report will be documented as a formal amendment to the final report.

In the absence of ongoing communications and after notification in writing to the Sponsor, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

Study Number: DSV/012
Enquiry Number: 29888B

Huntingdon Life Sciences

5 QUALITY ASSURANCE AND ARCHIVING PROCEDURES

5.1 Quality assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study Based Inspections	:	Critical phases of the study will be inspected.
Process based inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

5.2 Maintenance of records

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

APPENDIX 2

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2003

DSV012/042713

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

FR-1435X

WATER SOLUBILITY

Sponsor

Dead Sea Bromine Group
P.O. Box 180
Beer Sheva
84101
ISRAEL

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Final: 8 September 2004

CONTENTS

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

FR-1435X

Water Solubility

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8), as amended by EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

The stability of the test substance was the responsibility of the Sponsor.

P. Sydney ..... 8 September 2004

P. Sydney, B.Sc., M.Sc.
Study Director
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

FR-1435X

Water Solubility

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol Audit	8 March 2004	8 March 2004
Study Based Inspections		
Loading of support	22 April 2004	22 April 2004
Sample collection	23 April 2004	23 April 2004
Report Audit	13 May 2004	13 May 2004

In addition, process based inspections were conducted of other routine and repetitive procedures employed on this type of study at or about the time this study was in progress. Similarly an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were reported to Company Management.

H. Comb, B.Sc., M.R.Q.A.
Group Manager
Department of Quality Assurance
Huntingdon Life Sciences Ltd.

8 September 2004

Date

RESPONSIBLE PERSONNEL

FR-1435X

Water Solubility

The following staff member has reviewed this report.

A. L. Comb, B.Sc., Ph.D.
(Scientific Manager, Product Chemistry)

The following staff were responsible for the conduct of the work and reporting of the results.

P. Sydney, B.Sc., M.Sc.
(Study Director, Product Chemistry)

C. Steil, B.Sc.
(Scientist, Product Chemistry)

SUMMARY

A study was performed to determine the water solubility of FR-1435X.

The method followed is described in the EEC Methods for the determination of physicochemical properties (Method A6), specified in the Annex to Directive 92/69/EEC, the OECD Guidelines for the Testing of Chemicals (Method 105) and the EPA OPPTS Product Properties Test Guidelines (Method 830.7840).

FR-1435X was found to have a water solubility of 0.47 mg/l at 20°C.

INTRODUCTION

The study was performed to determine the water solubility of FR-1435X.

The protocol was approved by the Study Director and Huntingdon Life Sciences Management on 8 March 2004, and by the Sponsor on 9 March 2004.

The experimental start and completion dates were 9 March 2004 and 23 April 2004 respectively.

Location of study : Eye Research Centre
Eye
Suffolk
IP23 7PX

Primary data from the tests, and a copy of the final report, are stored in the archives of Huntingdon Life Sciences.

CONFIDENTIAL

TEST SUBSTANCE

Identity:	FR-1435X
[	]
Appearance:	Brown liquid
Storage conditions:	Room temperature
Lot number:	38278-53-4
Purity:	97.2%
Date received:	22 December 2003

WATER SOLUBILITY
(EEC Method A6, OECD Method 105, OPPTS Method 830.7840)

DEFINITION AND UNITS

The solubility in water is specified by the saturation mass concentration of the substance in water, and is a function of temperature. Solubility is specified in units of mass per volume of solution, and is reported as grams/litre (g/l).

PRELIMINARY TEST

A preliminary test was conducted by stirring approximately 10 mg of FR-1435X with 1000 ml of purified water for 7 days, and visually assessing dissolution. As undissolved material was apparent after this period, the solubility of FR-1435X was estimated to be less than 10 mg/l. The definitive test was subsequently performed by a flow through column elution method.

PREPARATION OF TEST SYSTEMS

A stainless steel column (25 cm x 4.6 mm internal diameter, fitted with a 2 µm frit) was packed with a sample (approximately 1 ml volume) of inert support material coated with the test substance:

Inert support material:	Gas chrom Q, 80-100 mesh (Phase Separations Ltd.)
Loading of test substance onto support:	Gas chrom Q (5 g) was mixed with a solution of the test substance in hexane (1 g/l, 100 ml). The solvent was removed under vacuum at 30°C to give 20 mg test substance/g support

The column was packed with a small glass wool plug between the frit and support.

A control column containing Gas chrom Q only was also prepared.

PROCEDURE

The charged column was connected to a HPLC pump equipped with stainless steel capillaries, and purified water was added to the solvent reservoir. The system was equilibrated at the test temperature of 20°C.

The flow through the column was started at 0.4 ml/minute, and the eluent allowed to run to waste for 18 hours before sampling commenced. Samples of the eluent (1 ml, measured gravimetrically to avoid unnecessary transfer stages) were then collected at intervals separated from each other by the passage of at least ten bed volumes. These were diluted to 2 ml with methanol for analysis by high performance liquid chromatography (HPLC).

After seven samples had been taken, the flow rate was reduced to 0.2 ml/minute and the eluent allowed to run to waste for an hour. A further seven samples were then collected and analysed as above.

On each sampling occasion at both flow rates, an additional sample was collected for pH measurement.

HPLC CONDITIONS

Instrument:	Hewlett Packard 1050 Liquid Chromatograph
Column:	Zorbax RX-C18 (25 cm x 4.6 mm internal diameter)
Column temperature:	Ambient
Mobile phase composition:	Methanol:water (9:1 v/v)
Flow rate:	1.2 ml/min
Injection volume:	100 µl
Detector:	UV set at 225 nm
Retention time:	The test substance chromatographed as two peaks, in a 1:1 ratio, at retention times of approximately 10 and 11 minutes. The summed areas of the peaks were used for quantification purposes.

PREPARATION OF CALIBRATION

A stock calibration solution of concentration 105.6 mg/l was prepared by weighing test substance (10.56 mg) into a 100 ml volumetric flask and dissolving in and diluting to volume with methanol.

Calibration solutions in the range 0.2 to 3 mg/l were prepared by dilutions of the stock solution with methanol:water (1:1 v/v).

CALCULATIONS

The concentration of FR-1435X in the analysed solution (C_A) was calculated from standards introduced before and after samples (bracketing standards) by the following equation:

$$C_A \text{ (mg/l)} = \frac{\text{sample peak area x standard concentration (mg/l)}}{\text{mean peak area of bracketing standards}}$$

The concentration of FR-1435X in the test solutions (C_B) was then calculated thus:

$$C_B \text{ (mg/l)} = C_A \text{ (mg/l)} \times \text{dilution factor}$$

where the dilution factor was 2 for all samples.

RESULTS AND DISCUSSION

The detector calibration was found to be linear over the range 0 to 3 mg/l of standard solutions in methanol:water (1:1 v/v) with a regression coefficient of 1.0000 (Table 1, Figure 1).

Table 2 presents a summary of the results of the test and shows that the water solubility of FR-1435X is 0.47 ± 0.01 mg/l. Table 3 presents the primary data for this test.

No peaks were observed in chromatograms of blank solutions, indicating that the analytical method was free from interference.

CONCLUSION

FR-1435X was found to have a water solubility of 0.47 mg/l at 20°C.

TABLE 1

Standard calibration for FR-1435X by HPLC

Standard concentration (mg/l)	Peak area
3.168	1306.9
2.534	1038.9
1.901	775.34
1.267	512.50
0.6336	256.71
0.3168	127.38
0.1584	63.643

Linear regression (including x = 0, y = 0) $y = 411.9x - 3.567$, $r = 1.0000$

x = concentration
y = peak area

FIGURE 1

Standard calibration for FR-1435X by HPLC

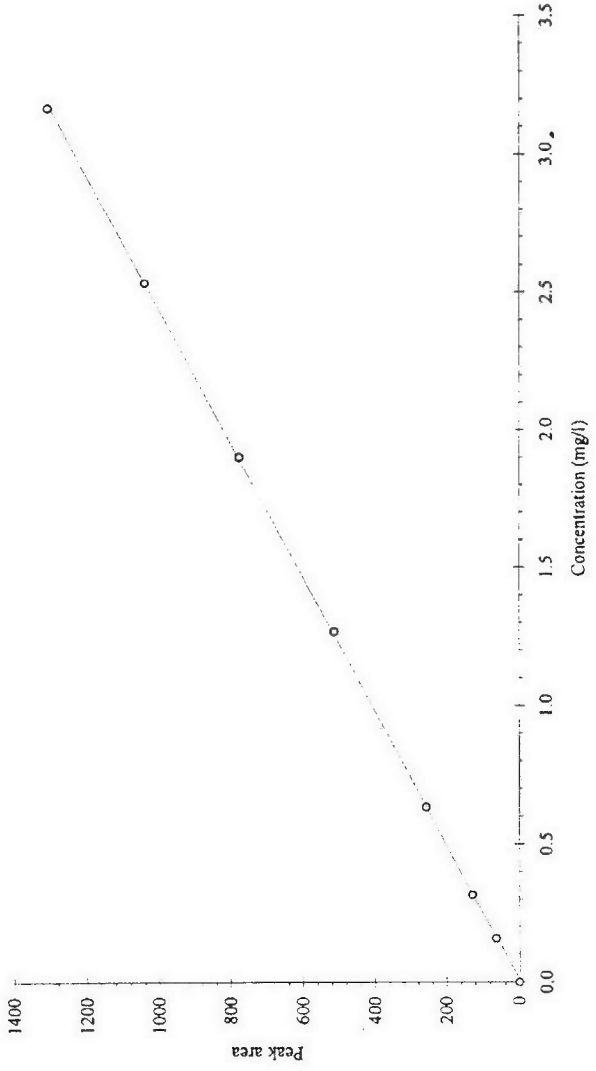

TABLE 2

Measurements of water solubility at 20°C

1. Column flow rate 0.4.ml/minute

Sample number	Sampling time (h)	Concentration (mg/l)	pH
1	18	0.465	6.44
2	18.5	0.460	6.50
3	19	0.462	6.46
4	19.5	0.473	6.36
5	20	0.473	6.43
6	20.5	0.462	6.42
7	21	0.472	6.55

Mean solubility = 0.467 ± 0.006 mg/l (C. of V. = 1.3%)

2. Column flow rate 0.2 ml/minute

Sample number	Sampling time (h)	Concentration (mg/l)	pH
8	22	0.471	6.46
9	22.5	0.470	6.45
10	23	0.470	6.46
11	23.5	0.479	6.44
12	24	0.478	6.53
13	24.5	0.474	6.48
14	25	0.470	6.53

Mean solubility = 0.473 ± 0.004 mg/l (C. of V. = 0.9%)

Overall mean solubility = 0.470 ± 0.006 mg/l (C. of V. = 1.3%)

TABLE 3

HPLC analysis of samples from the water solubility test for FR-1435X

Sample	Peak area	C_A (mg/l)	Dilution factor	C_B (mg/l)
0.6336 mg/l std	256.36	-	-	-
Sample 1	93.975	0.2326	2	0.465
Sample 2	92.878	0.2299	2	0.460
Sample 3	93.373	0.2311	2	0.462
0.6336 mg/l std	255.62	-	-	-
Sample 4	95.451	0.2365	2	0.473
Sample 5	95.506	0.2366	2	0.473
Sample 6	93.130	0.2307	2	0.462
0.6336 mg/l std	255.84	-	-	-
Sample 7	95.103	0.2360	2	0.472
Sample 8	94.931	0.2356	2	0.471
Sample 9	94.688	0.2350	2	0.470
0.6336 mg/l std	254.71	-	-	-
Sample 10	94.630	0.2349	2	0.470
Sample 11	96.564	0.2397	2	0.479
Sample 12	96.334	0.2391	2	0.478
0.6336 mg/l std	255.77	-	-	-
Sample 13	95.570	0.2370	2	0.474
Sample 14	94.712	0.2349	2	0.470
0.6336 mg/l std	255.23	-	-	-

APPENDIX 1

STUDY PROTOCOL

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

CONFIDENTIAL

PROTOCOL

FR-1435X

WATER SOLUBILITY

Sponsor

Dead Sea Bromine Group
P.O. Box 180
Beer Sheva
84101
ISRAEL

Research Laboratory

Huntingdon Life Sciences Limited
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 12

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

CONTACT DETAILS

Sponsor:	Dr Sarit Lifshitz
Address:	Dead Sea Bromine Group P.O. Box 180 Beer Sheva 84101 ISRAEL
Telephone No:	(972)-8-6297672
Fax No:	(972)-8-6297832
E-mail:	Lifshitzs@dsbg.com

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

PROTOCOL APPROVAL

FR-1435X

WATER SOLUBILITY

P. Sydney

8 March 2004

P. Sydney, B.Sc., M.Sc.
Study Director
Huntingdon Life Sciences Ltd

Date

A. L. Comb

8 March 2004

A. L. Comb, B.Sc., Ph.D.
Management
Huntingdon Life Sciences Ltd

Date

Sarit Lifshitz

9.3.04

Sponsor
Dead Sea Bromine Group

Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

PLEASE NOTE THAT THE STUDY CANNOT BEGIN UNLESS HUNTINGDON LIFE SCIENCES IS IN RECEIPT OF A PROTOCOL APPROVAL SIGNED BY THE SPONSOR

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

FR-1435X

WATER SOLUBILITY

Number of pages for internal distribution: 9

This working document is approved for circulation and use:

P. Sydney

8 March 2004

P. Sydney, B.Sc., M.Sc.
Study Director

Date

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

CONTENTS

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

1. INTRODUCTION

1.1 Objective and reason for study

Information on physicochemical properties is important in the assessment of the potential hazards of a substance during manufacture, transportation and storage, is useful in the characterisation of the material and aids in the prediction if its environmental fate. In addition, knowledge of certain physicochemical properties is useful for performing and interpreting toxicological and ecotoxicological studies.

1.2 Regulatory acceptance

The study is designed in accordance with accepted principles in order to meet the requirements of the Annex to European Commission Directive 92/69/EEC, the OECD Guidelines for Testing of Chemicals and EPA Product Properties Test Guidelines (OPPTS).

1.3 Good Laboratory Practice

The study will be conducted in compliance with the principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

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Huntingdon Life Sciences

2. STUDY DETAILS

2.1 Study Director

P. Sydney, B.Sc., M.Sc.

In the temporary absence of the Study Director, routine duties and technical queries will be attended to by A. L. Comb; formal GLP responsibilities will be assumed by Management.

2.2 Testing laboratory

Name:	Huntingdon Life Sciences Limited
Address:	Department of Product Chemistry, Eye Research Centre, Eye, Suffolk, IP23 7PX
Telephone:	+44 (0) 1379 672033
Facsimile:	+44 (0) 1379 672471
E-Mail:	SydneyP@UKOrg.Huntingdon.com

2.3 Study schedule

Study No.:	DSV/011
Experimental start:	March 2004
Completion:	April 2004
Despatch of draft report:	May 2004

The actual dates will be detailed in the final report.

2.4 Health and Safety

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work Act 1974, and the current Control of Substances Hazardous to Health Regulations, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

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Huntingdon Life Sciences

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

CONFIDENTIAL

2.5 Test substance

Identity:	FR-1435X
[	]
Appearance:	Brown liquid
Storage conditions:	Room temperature
Lot number:	38278-53-4
Expiry:	To be included in the raw data if/when available
Purity:	97.2%

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

3. EXPERIMENTAL PROCEDURE

3.1 Method

A flow through column elution method (EEC Method A6, OECD Test Guideline 105, OPPTS 830.7840) will be employed.

3.2 Definition and units

The solubility in water of a substance is defined by the saturation mass concentration of the substance in water and is a function of temperature.

Solubility is specified in units of weight per volume of solution and will be reported as grams/litre (g/l).

3.3 Preliminary test

A known weight of test substance will be shaken with increasing volumes of purified water in a stepwise manner and dissolution will be assessed visually. The solubility of the test substance will be estimated from the observations. Additionally, an excess of test substance may be stored with water over an extended time period in order to establish a more reliable estimate.

3.4 Main test

The test substance will be loaded on an inert carrier to give 1-10% (w/w) of test substance. A weighed amount of test substance will be dissolved in a suitable solvent and an appropriate weight of carrier added. The solvent will be completely removed using a rotary evaporator.

One test column and one "blank" column containing no test substance will then be prepared. The column used will be stainless steel with an appropriate end-fitting containing a frit, and will be filled with the coated packing. The charged column is then connected to an HPLC pump equipped with stainless steel capillaries. The system will be equilibrated at the test temperature of 20°C ± 0.5°C.

The appropriate aqueous media will be added to the solvent reservoir and the flow through the column will be started. At least the first five bed volumes of water will be discarded from the column. The system will then be run until equilibration is established as defined by five successive test column samples whose concentrations do not differ by more than ±30% in a random fashion. The samples should be separated from each other by time intervals corresponding to the passage of at least ten bed volumes of the eluent.

The samples will be examined visually for any Tyndall effect (light scattering) and rejected if this effect is observed. The pH of each sample of saturated solution will be determined. The concentration of test substance in the saturated solution will be determined using a suitable substance specific analytical method, following extraction and concentration of the samples if required. If the extraction and concentration step is necessary, this will be conducted immediately on sampling. Aqueous samples will not be stored.

The procedure will then be repeated on the packed column using half the flow rate of the first. If the results of the runs at the two flows are in agreement, the test is satisfactory; if there is a higher apparent solubility with the lower flow rate, then the halving of the flow rates will continue until two successive runs give the same solubility.

3.5 Treatment of results

The mean value from at least five consecutive samples differing by no more than ±30% will be determined. The results obtained from the two columns should not differ by more than 30% otherwise a longer equilibration period will be used. The standard deviation will also be calculated.

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

4. REPORTING

The results of these studies will be reported to the Sponsor to include, but not necessarily be limited to, the following:

The determined water solubility value for each sample, the mean value and the standard deviation.

For compounds that decompose at a rate such that a precise value for the water solubility cannot be obtained, a statement to that effect.

For compounds with a water solubility below 1 ppb a statement that the water solubility is less than 1 ppb.

A complete description of the analytical method used.

The extracting solvent used, and its extracting efficiency.

Any changes made or problems encountered in the test procedure.

Two types of report are issued:

Draft report:	After QA audit, for review by the Sponsor
Final report:	After approval by the Sponsor

Routinely, reports are supplied on A4 paper and the following number are supplied:

Draft report:	1 unbound
Final report	1 bound (with original signature pages) and 1 unbound

Any additions or corrections to an authorised final report will be documented as a formal amendment to the final report.

In the absence of ongoing communications and after notification in writing to the Sponsor, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

Study Number: DSV/011
Enquiry Number: 29888A

Huntingdon Life Sciences

5 QUALITY ASSURANCE AND ARCHIVING PROCEDURES

5.1 Quality assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study Based Inspections	:	Critical phases of the study will be inspected.
Process based inspections	:	Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.
Report Audit	:	The draft report and study data will be audited before issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

5.2 Maintenance of records

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

APPENDIX 2

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2003

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

FR-1435X

VALIDATION OF METHOD OF ANALYSIS IN AQUEOUS SOLUTION

Sponsor

Dead Sea Bromine Group
P.O. Box 180
Beer Sheva
84101
ISRAEL

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Final: 22 July 2004

CONTENTS

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

FR-1435X

Validation of Method of Analysis in Aqueous Solution

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8), as amended by EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

The stability of the test substance was the responsibility of the Sponsor.

P. Sydney ..... 22 July 2004

P. Sydney, B.Sc., M.Sc.
Study Director
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

FR-1435X

Validation of Method of Analysis in Aqueous Solution

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol Audit	26 February 2004	26 February 2004
Study Based Inspections Fortification of test media	26 - 29 March 2004	31 March 2004
Report Audit	23 April 2004	26 April 2004

In addition, process based inspections were conducted of other routine and repetitive procedures employed on this type of study at or about the time this study was in progress. Similarly an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were reported to Company Management.

H. Comb, B.Sc., M.R.Q.A.
Group Manager
Department of Quality Assurance
Huntingdon Life Sciences Ltd.

22 July 2004
Date

RESPONSIBLE PERSONNEL

FR-1435X

Validation of Method of Analysis in Aqueous Solution

The following staff member has reviewed this report.

A. L. Comb, B.Sc., Ph.D.
(Scientific Manager, Product Chemistry)

The following staff were responsible for the conduct of the work and reporting of the results.

P. Sydney, B.Sc., M.Sc.
(Study Director, Product Chemistry)

C. Steil, B.Sc.
(Scientist, Product Chemistry)

SUMMARY

The study was designed to develop and validate a method of analysis for the determination of the concentration of FR-1435X in various aqueous media. High performance liquid chromatography (HPLC) with ultraviolet (UV) detection was employed.

The method was validated with respect to linearity, accuracy, precision, range and specificity.

The method was demonstrated to be linear, and suitably accurate and precise, to enable determination of FR-1435X at a range of concentrations in octanol saturated water, dechlorinated water, Elendt M4 medium and algal medium, and was found to be free of interference from any of the test media investigated.

INTRODUCTION

The study was designed to develop and validate a method of analysis for the determination of the concentration of FR-1435X in various aqueous media.

The protocol was approved by the Study Director, Huntingdon Life Sciences Management and the Sponsor on 24 February 2004.

The experimental start and completion dates were 27 February 2004 and 1 April 2004 respectively.

Location of study : Eye Research Centre
Eye
Suffolk
IP23 7PX

Primary data from the tests, and a copy of the final report, are stored in the archives of Huntingdon Life Sciences.

TEST SUBSTANCE

Identity:	FR-1435X
[	]
Appearance:	Brown liquid
Storage conditions:	Room temperature
Lot number:	38278-53-4
Purity:	97.2%
Date received:	22 December 2003

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TEST MEDIA

Samples of the following test media were supplied by the Departments of Product Chemistry and Ecotoxicology at Huntingdon Life Sciences:

Octanol saturated water (as used for partition coefficient studies)

Dechlorinated water (as used for acute toxicity to trout studies)

Elendt M4 (as used for acute toxicity to *Daphnia* studies)

Algal medium (as used for algal growth inhibition studies)

METHOD VALIDATION

Analytical method

A method of analysis was developed for the determination of the concentration of FR-1435X in various aqueous media. The method was based on high performance liquid chromatography (HPLC) employing ultraviolet (UV) detection.

The following HPLC conditions were found suitable.

Instrument:	Hewlett Packard 1050 Liquid Chromatograph
Column:	Zorbax RX-C18 (25 cm x 4.6 mm internal diameter)
Column temperature:	Ambient
Mobile phase composition:	Methanol:water (9:1 v/v)
Flow rate:	1.2 ml/min
Injection volume:	100 µl
Detector:	UV set at 225 nm
Retention time:	The test substance chromatographed as two peaks, in a 1:1 ratio, at retention times of approximately 10 and 11 minutes. The summed areas of the peaks were used for quantification purposes.

Instrument response – linearity and precision

The analytical method was examined in order to determine the linearity of the detector response with respect to the concentration of FR-1435X over the working range of the method.

A stock calibration solution of concentration 1040 mg/l was prepared by weighing test substance (26.01 mg) into a 25 ml volumetric flask and dissolving in and diluting to volume with methanol.

Calibration solutions in the ranges 0.004 to 0.05, 0.05 to 2 and 0.2 to 10 mg/l were prepared by dilutions of the stock solution with methanol:water (1:1 v/v). These were chromatographed using the conditions described. Calibration graphs of concentration versus peak area were prepared, and the data were regressed linearly (Tables 1 to 3, Figures 1 to 3). The calibrations were found to be linear over the ranges investigated, with correlation coefficients of ≥ 0.999.

The limit of detection (LOD), defined as the minimum concentration of analyte in the test medium which would produce an instrumental response three times that of local baseline noise after sample processing, was estimated to be approximately 0.007 mg/l.

The limit of quantitation (LOQ), defined as the minimum concentration of analyte in the test medium which would produce an instrumental response ten times that of local baseline noise after sample processing, was estimated to be approximately 0.022 mg/l.

Triplicate injections of the highest working calibration solution showed good precision (RSD = 0.2%).

Recovery experiments – accuracy, precision and range

Samples of each test medium were fortified according to the following scheme:

	Fortification (mg/l)		
	LOQ	1	10
Octanol saturated water	x3	x2	-
Dechlorinated water	x3	x2	x2
Elendt M4	-	x2	-
Algal medium	-	x2	-

The fortified samples were prepared by addition of stock solutions of the test substance in methanol, such that the concentrations in each test medium indicated above were achieved with 1% v/v of the co-solvent present. The fortified samples (10 ml) were diluted to 20 ml with methanol for analysis by HPLC.

The concentration of FR-1435X in the analysed solution (C_A) was calculated from standards introduced before and after samples (bracketing standards) by the following equation:

$$C_A \text{ (mg/l)} = \frac{\text{sample peak area x standard concentration (mg/l)}}{\text{mean peak area of bracketing standards}}$$

The accuracy of the method was then determined as the percentage recovery (R) from the fortified samples as follows:

$$\text{Recovery (\%)} = \frac{C_A \text{ x D x 100}}{F}$$

where D = dilution factor (2)
F = concentration of fortified sample (mg/l)
100 = conversion to percent

Table 4 presents the raw data from the recovery experiments. Table 5 shows the mean recoveries (accuracy) and the relative standard deviations (precision) achieved for each test medium. Recoveries were essentially quantitative over the range of the method.

Specificity

Example sample chromatograms for each of the test media fortified at the 1 mg/l level are shown in Figures 4 to 7, whilst chromatograms of unfortified samples are presented in Figures 8 to 11. No significant interfering peaks were evident from any of the media, and the method is therefore considered specific for the analyte in these matrices. Typical standard chromatograms (1 and 0.01 mg/l) are shown in Figures 12 and 13.

TABLE 1

Standard calibration for FR-1435X by HPLC (0.004 to 0.05 mg/l)

Standard concentration (mg/l)	Peak area
0.05202	16.086
0.02497	8.1543
0.02081	6.4062
0.01665	4.9564
0.01248	3.9826
0.008323	2.5180
0.004162	1.2642

Linear regression (including x = 0, y = 0) $y = 311.1x - 0.0009762$, $r = 0.9994$

x = concentration
y = peak area

FIGURE 1

Standard calibration for FR-1435X by HPLC (0.004 to 0.05 mg/l)

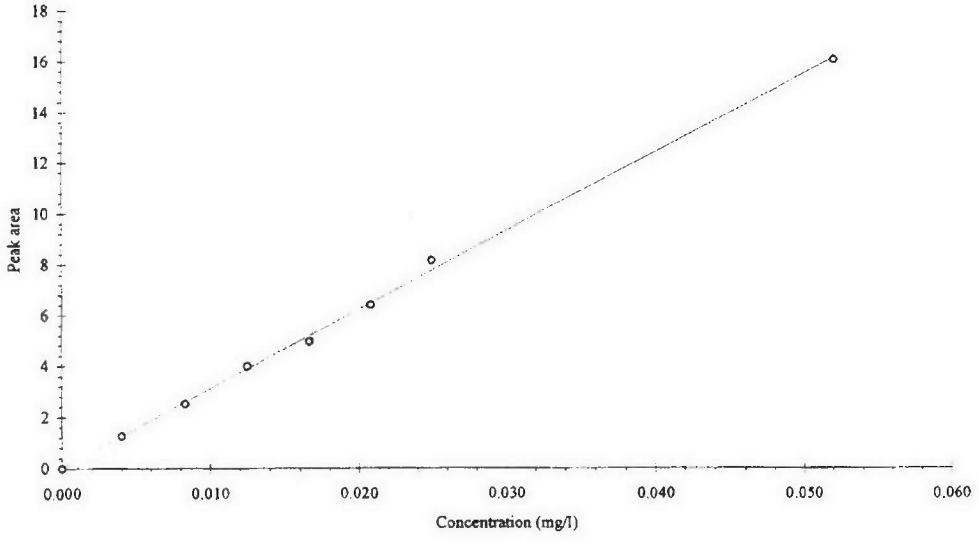


TABLE 2

Standard calibration for FR-1435X by HPLC (0.05 to 2 mg/l)

Standard concentration (mg/l)	Peak area
2.081	815.77
1.040	417.56
0.2081	82.414
0.1040	41.451
0.05202	23.271

Linear regression (including x = 0, y = 0) $y = 392.7x + 1.978$, $r = 0.9999$

x = concentration
y = peak area

FIGURE 2

Standard calibration for FR-1435X by HPLC (0.05 to 2 mg/l)

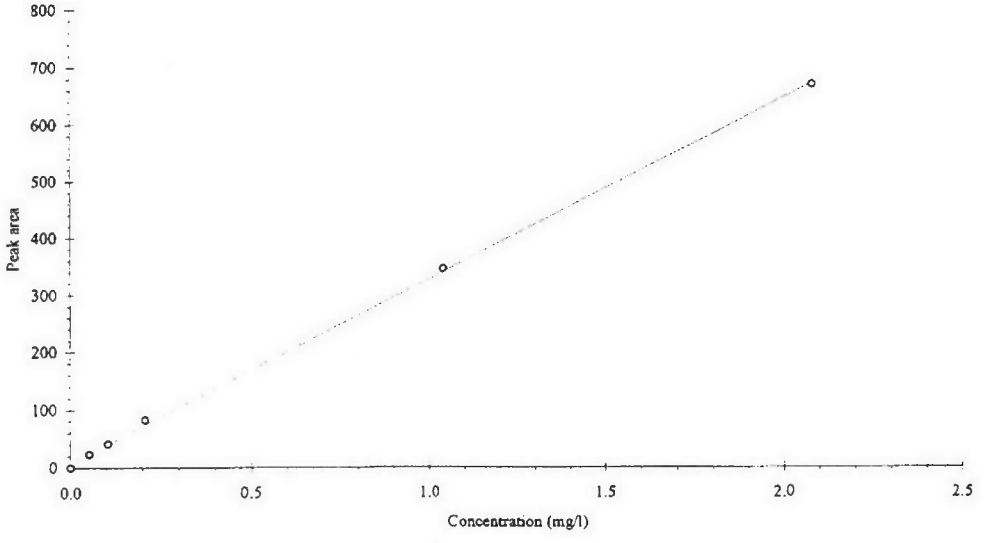


TABLE 3

Standard calibration for FR-1435X by HPLC (0.2 to 10 mg/l)

Standard concentration (mg/l)	Peak area
10.40	4117.5
5.202	2021.0
2.081	815.77
1.040	417.56
0.5202	199.13
0.2081	82.414

Linear regression (including x = 0, y = 0) $y = 395.0x - 4.171$, $r = 1.0000$

x = concentration
y = peak area

FIGURE 3

Standard calibration for FR-1435X by HPLC (0.2 to 10 mg/l)

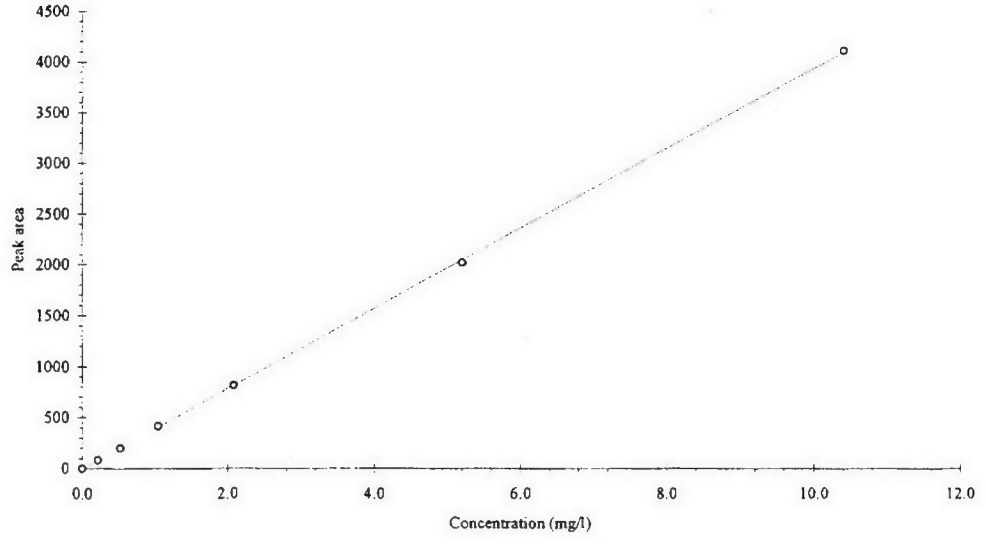


TABLE 4

HPLC analysis of fortified samples of aqueous media for FR-1435X

Sample	Peak area	C_A (mg/l)	Dilution factor	Concentration of fortified solution (mg/l)	Recovery (%)
0.01248 mg/l std	4.1402	-	-	-	-
Octanol/LOQ/A	3.5843	0.01119	2	0.02081	108
Octanol/LOQ/B	3.9940	0.01247	2	0.02081	120
Octanol/LOQ/C	4.0409	0.01261	2	0.02081	121
0.01248 mg/l std	3.8570	-	-	-	-
Dechlor/LOQ/A	3.8541	0.01219	2	0.02081	117
Dechlor/LOQ/B	3.4707	0.01098	2	0.02081	106
Dechlor/LOQ/C	3.8856	0.01229	2	0.02081	118
0.01248 mg/l std	4.0345	-	-	-	-
0.5202 mg/l std	199.33	-	-	-	-
Octanol/1/A	208.31	0.5454	2	1.040	105
Octanol/1/B	209.63	0.5489	2	1.040	106
Dechlor/1/A	198.51	0.5198	2	1.040	100
Dechlor/1/B	196.61	0.5148	2	1.040	99
0.5202 mg/l std	198.01	-	-	-	-
Elendt/1/A	202.22	0.5300	2	1.040	102
Elendt/1/B	187.52	0.4915	2	1.040	95
Algal/1/A	199.38	0.5226	2	1.040	100
Algal/1/B	195.94	0.5136	2	1.040	99
0.5202 mg/l std	198.92	-	-	-	-
5.202 mg/l std	1978.4	-	-	-	-
Dechlor/10/A	2024.9	5.303	2	10.40	102
Dechlor/10/B	2007.7	5.258	2	10.40	101
5.202 mg/l std	1994.6	-	-	-	-

TABLE 5

Accuracy and precision of the method

Test medium	Fortification level (mg/l)	Recovery (%)	Mean recovery (%)	RSD (%)
Octanol saturated water	0.021	108, 120, 121	116	6.5
	1	105, 106	106	0.5
Dechlorinated water	0.021	117, 106, 118	114	6.2
	1	100, 99	100	0.7
	10	102, 101	102	0.6
Elendt M4 medium	1	102, 95	99	5.3
Algal medium	1	100, 99	100	1.2

FIGURE 4

Chromatogram of a fortified (1 mg/l) octanol saturated water sample

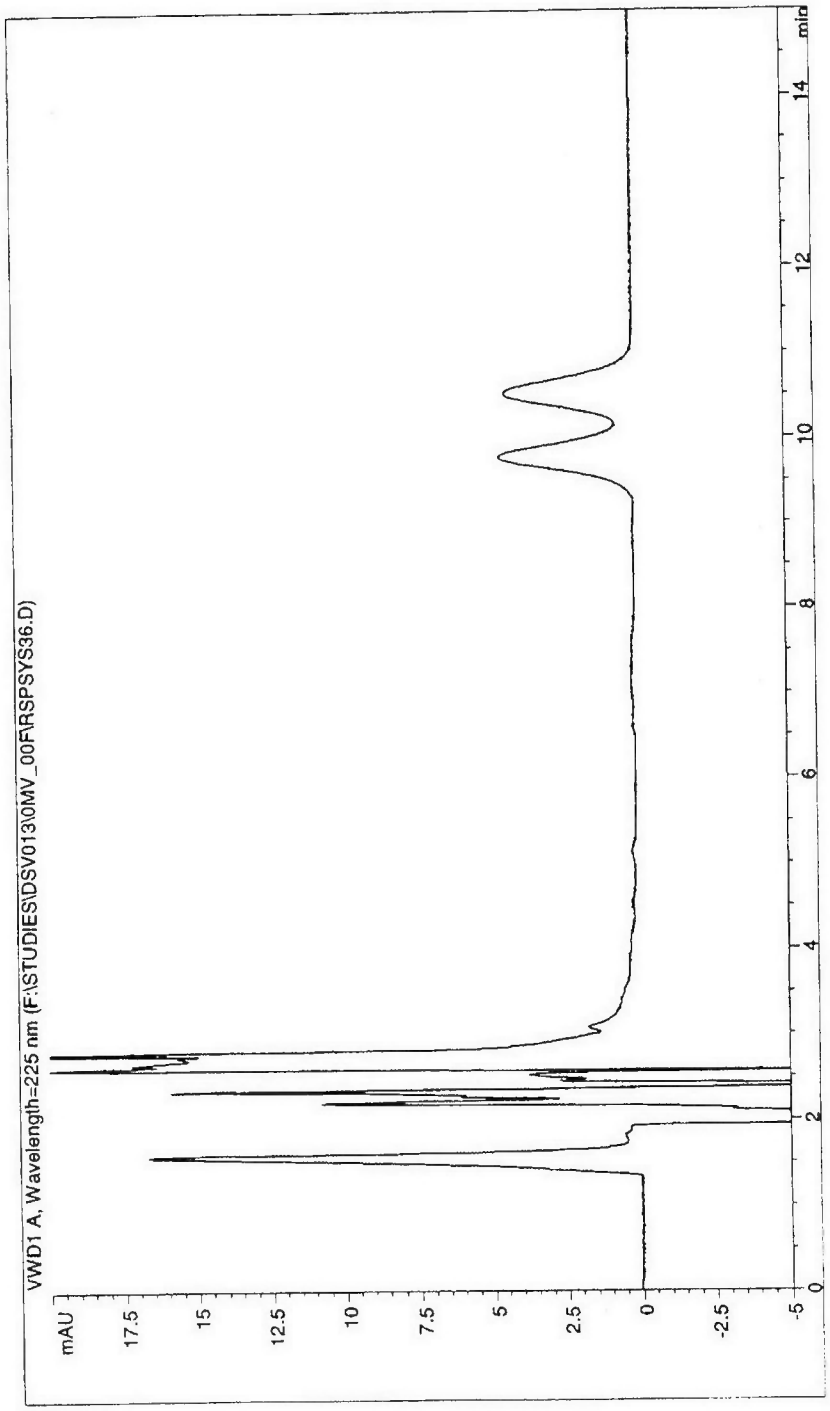


FIGURE 5

Chromatogram of a fortified (1 mg/l) dechlorinated water sample

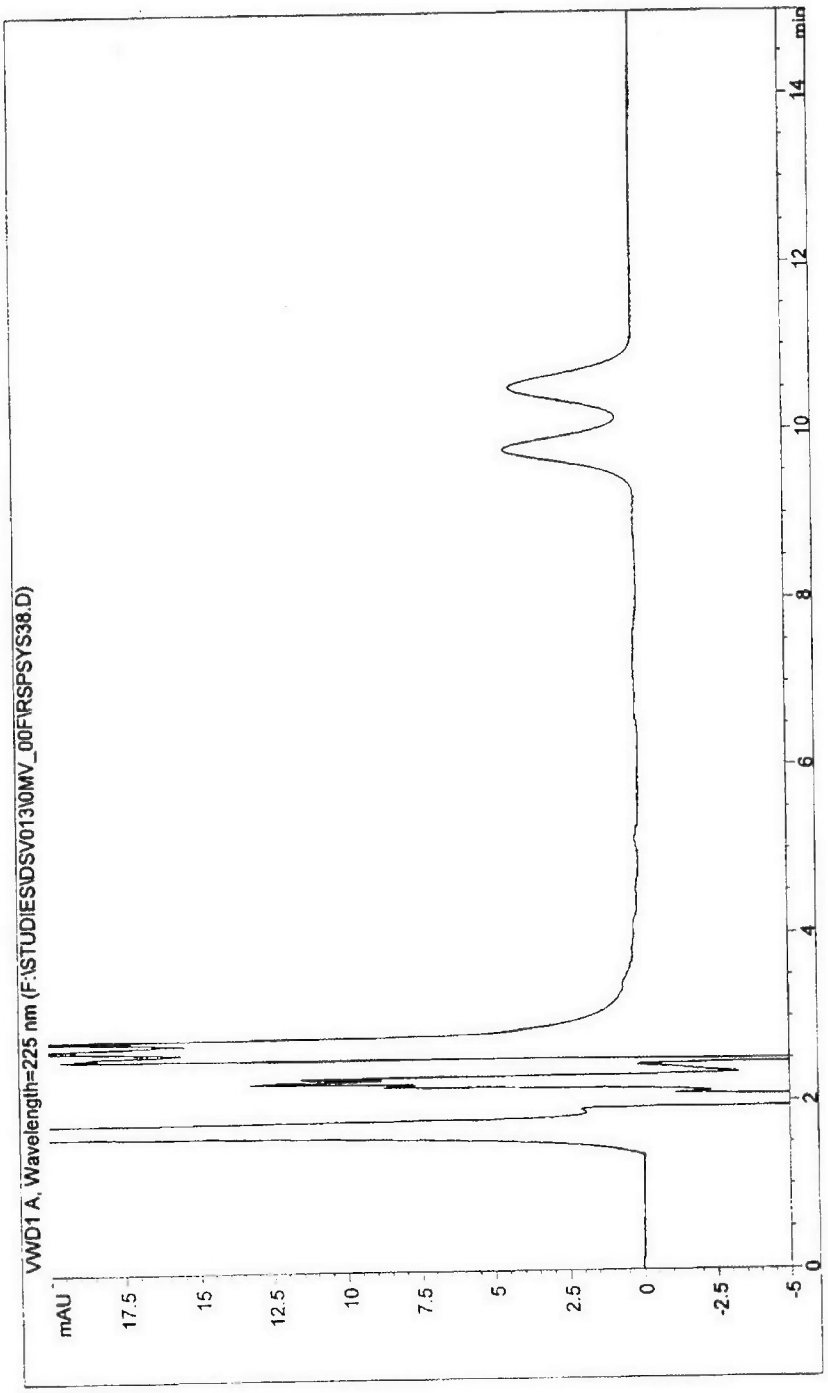


FIGURE 6

Chromatogram of a fortified (1 mg/l) Elendt M4 medium sample

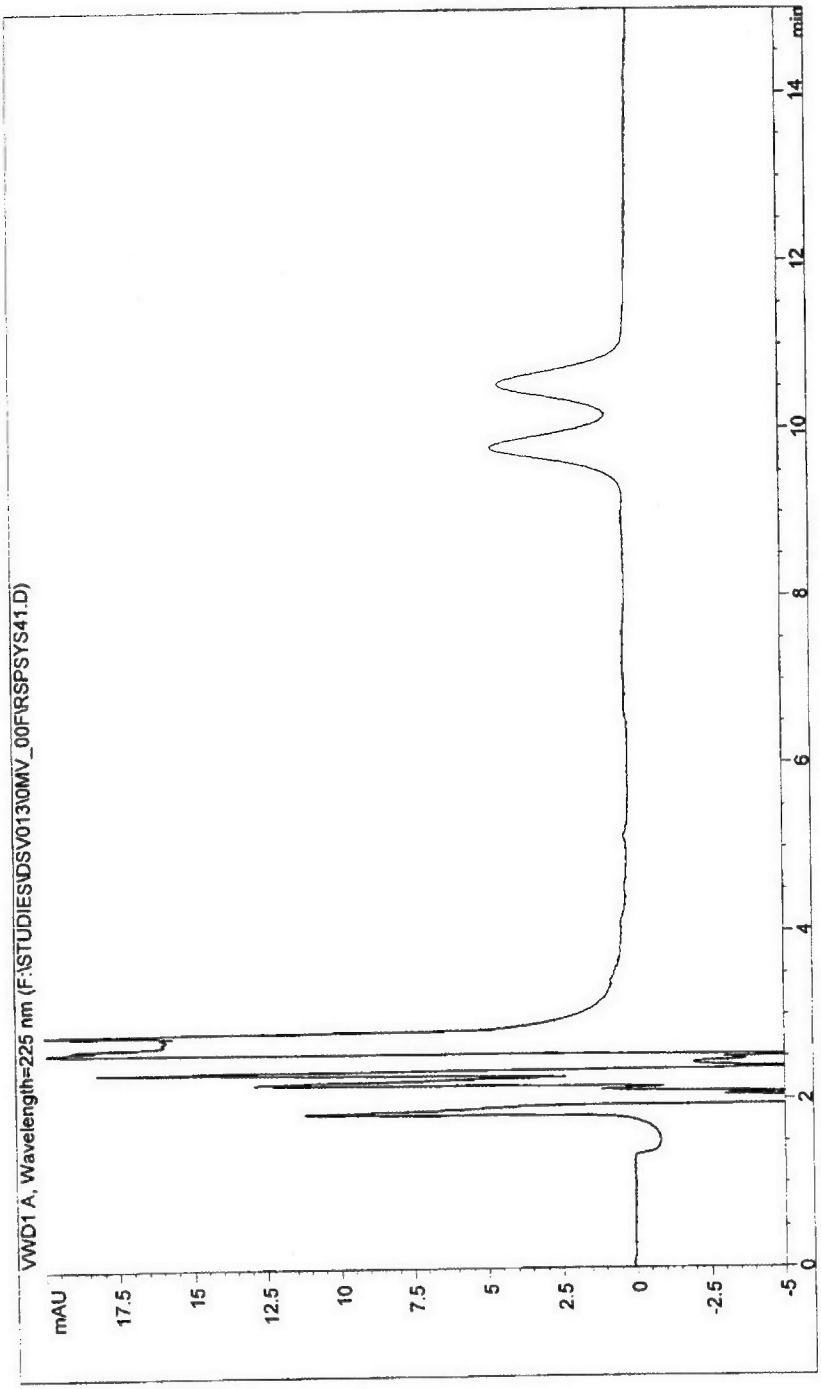


FIGURE 7

Chromatogram of a fortified (1 mg/l) algal medium sample

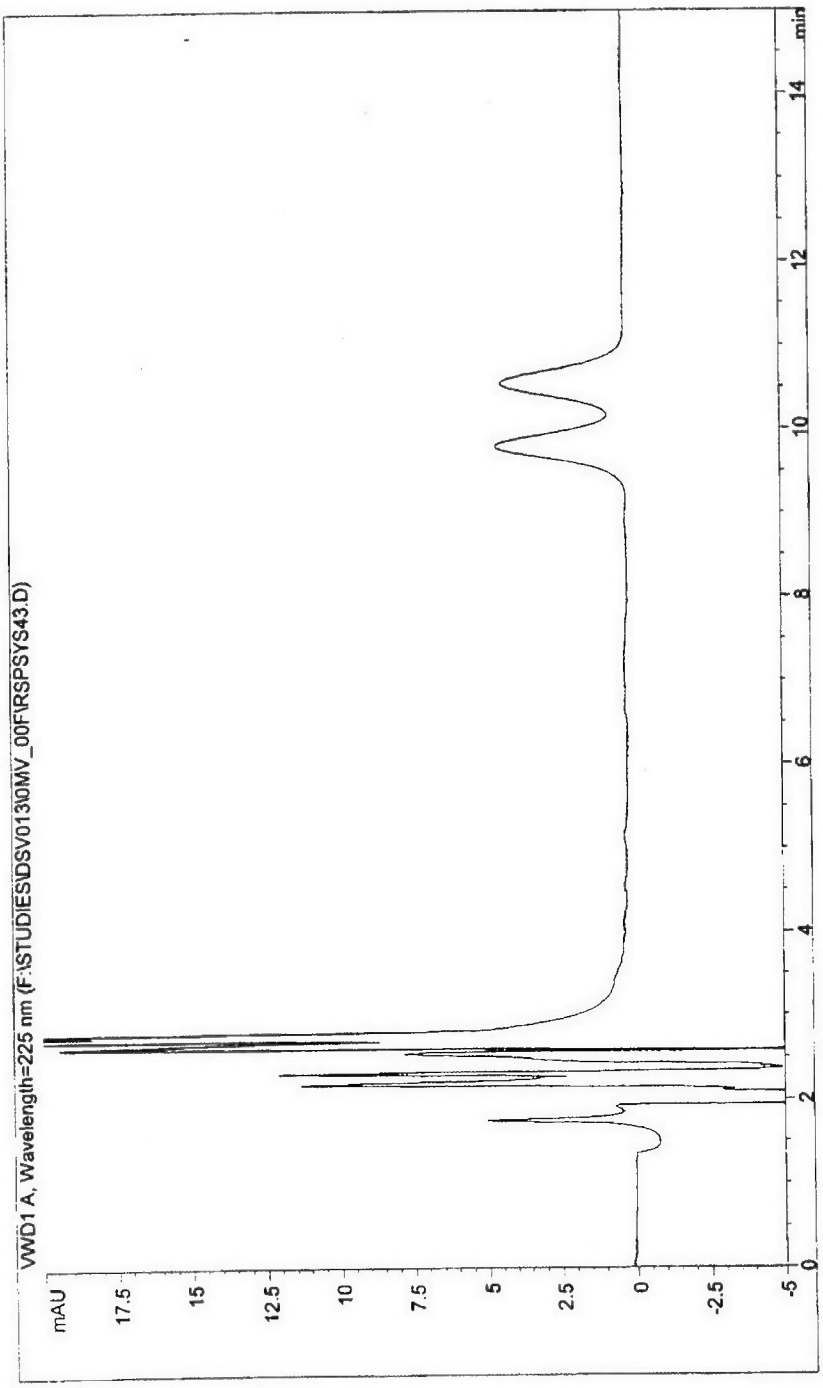


FIGURE 8

Chromatogram of unfortified octanol saturated water

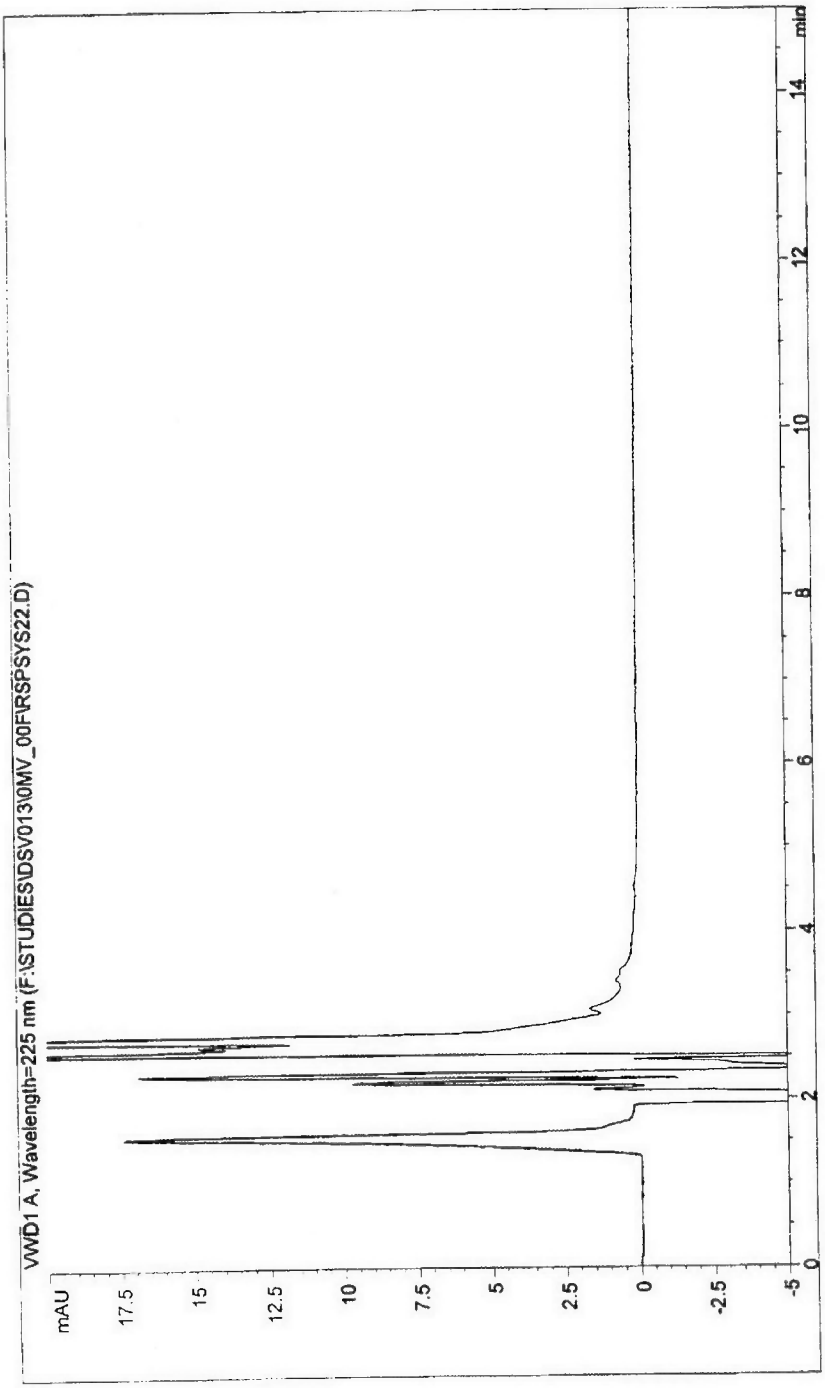


FIGURE 9

Chromatogram of unfortified dechlorinated water

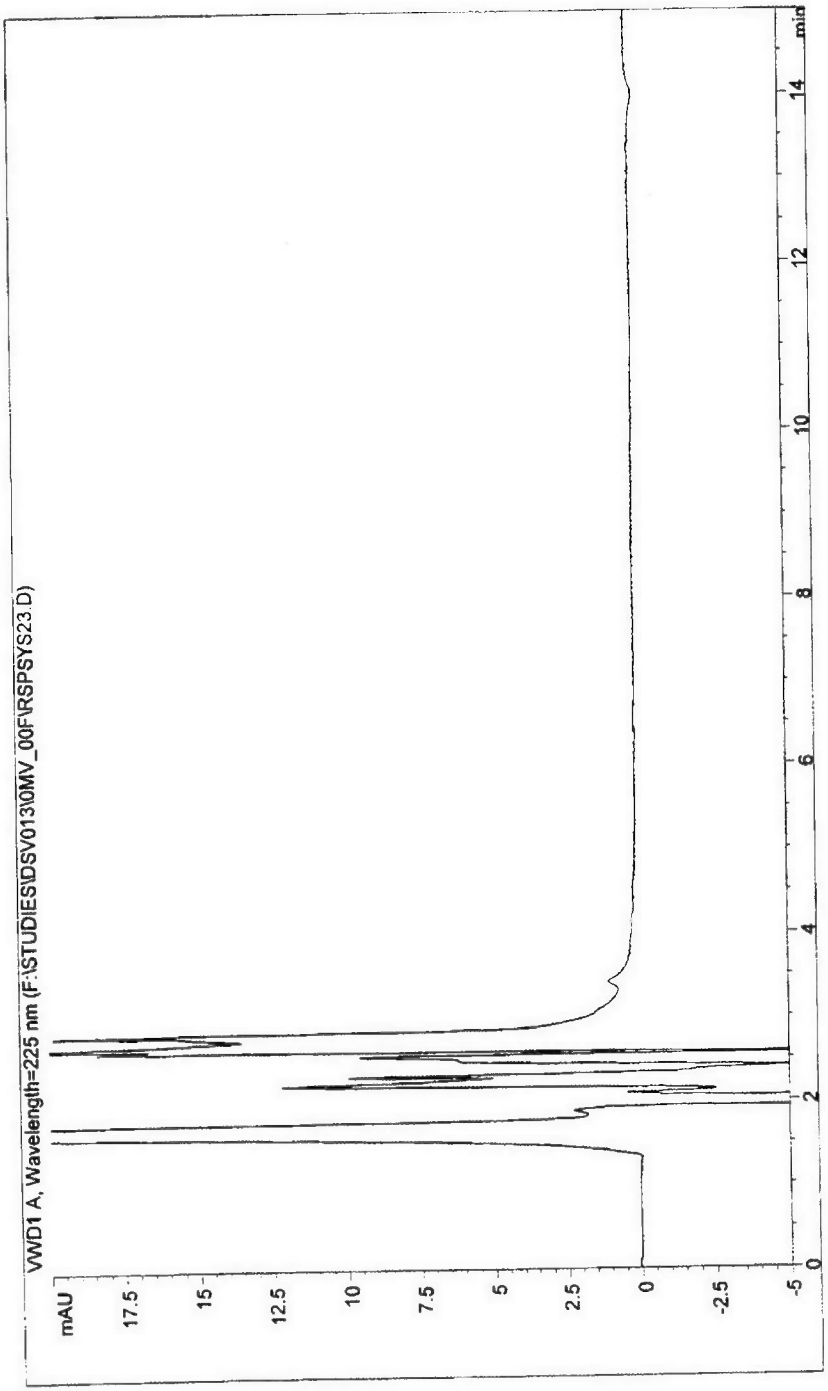


FIGURE 10

Chromatogram of unfortified Elendt M4 medium

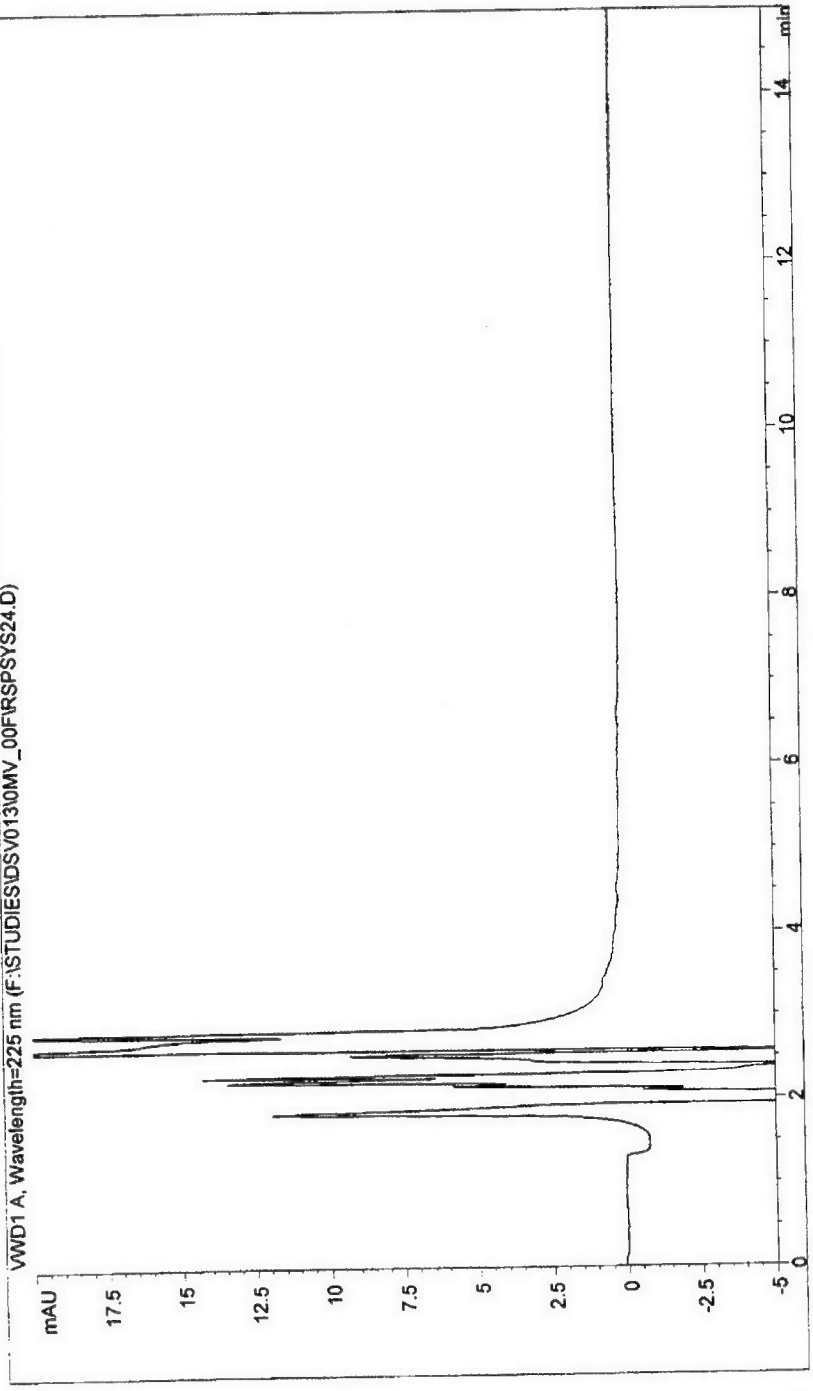


FIGURE 11

Chromatogram of unfortified algal medium

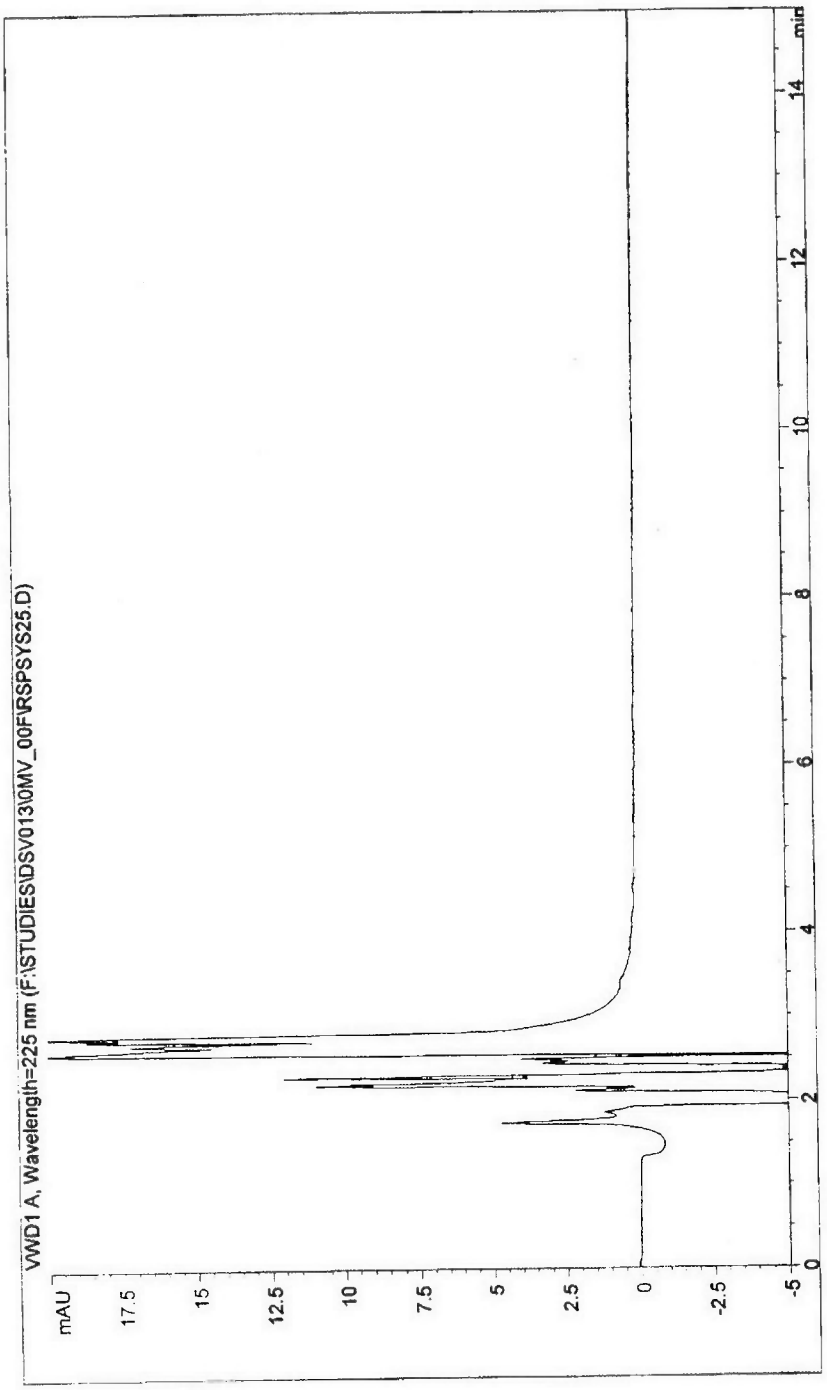


FIGURE 12

Typical FR-1435X standard chromatogram (1 mg/l)

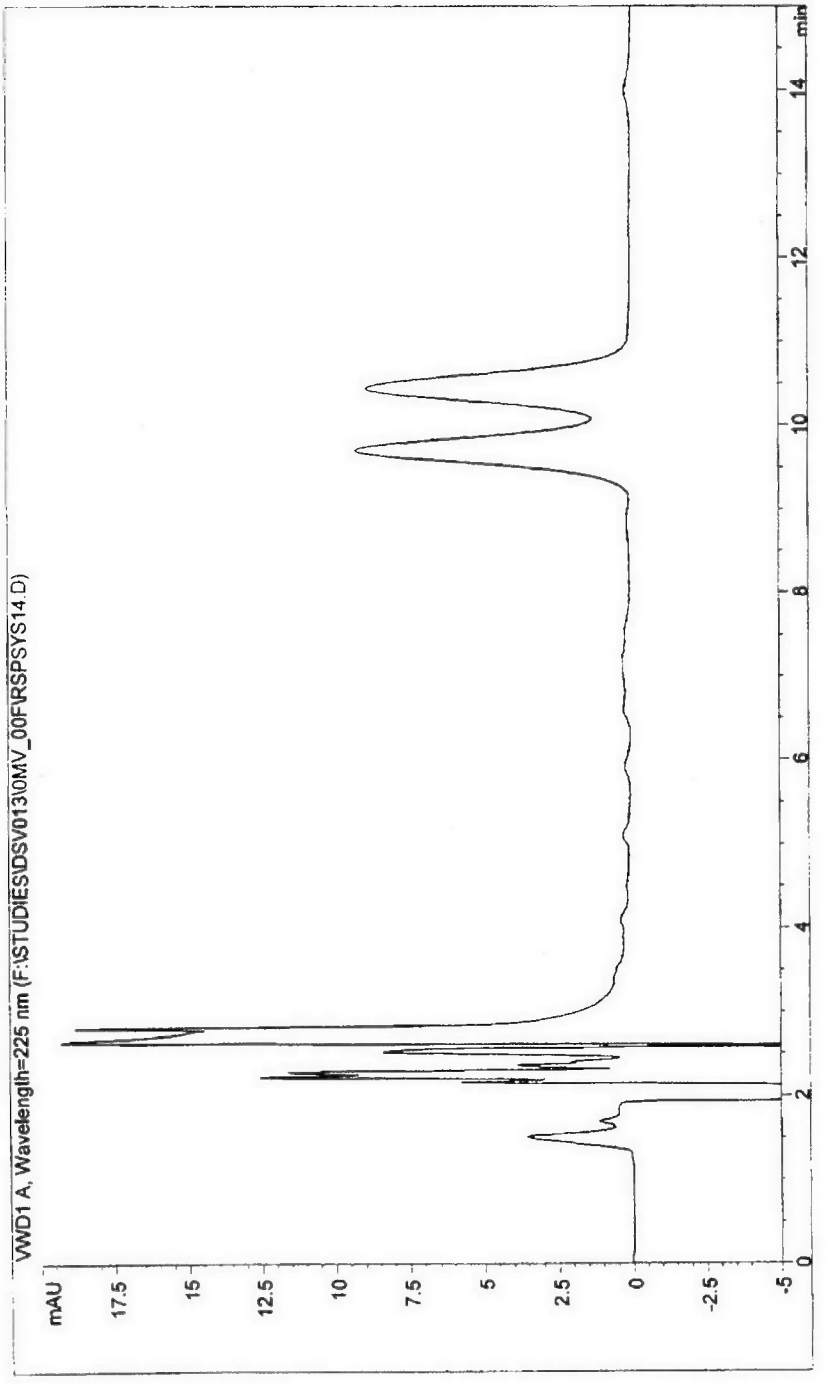
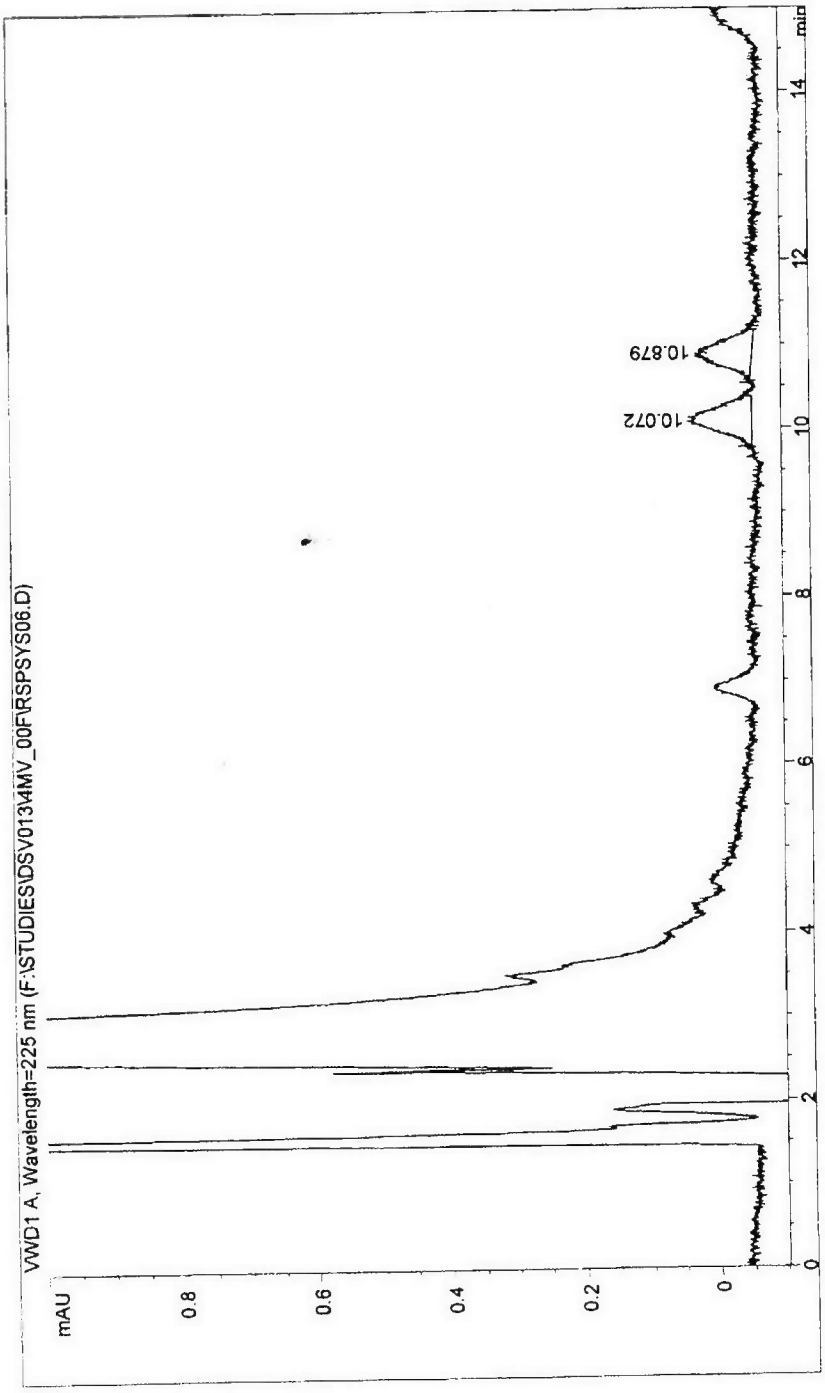


FIGURE 13

Typical FR-1435X standard chromatogram (0.01 mg/l)



APPENDIX 1

EYE RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2003

GLP

DEPARTMENT OF HEALTH

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

- Attachment 8 -

REPORT

Study Title

PRIMARY SKIN IRRITATION/CORROSION STUDY WITH FR-1435X IN THE RABBIT (4-HOUR SEMI-OCCLUSIVE APPLICATION)

Author

Drs. A.H.B.M. van Huygevoort

Study completion date

02 June 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419873
NOTOX Substance 146232/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

NOTOX B.V.

Drs. A.H.B.M. van Huygevoort
Study Director

Date: 2 June 2005

Drs. M.S. Teunissen
Section Head Toxicology

Date: 09 June 2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	protocol	12-Oct-04	12-Oct-04	12-Oct-04
	Report	04-Mar-05	04-Mar-05	07-Mar-05
Process	**SPF unit**	1-Nov-04	11-Nov-04	11-Nov-04
	Test substance handling			
	Exposure			
	Observation/Measurement			
	Specimen handling			

Head of Quality Assurance
C.J.Mitchell B.Sc.

I.A.

Date: 13 JAN 2005

4. SUMMARY

Primary skin irritation/corrosion study with FR-1435X in the rabbit (4-hour semi-occlusive application).

The study was carried out based on the guidelines described in: OECD No.404, "Acute Dermal Irritation/Corrosion" (2002); EC Commission Directive 2004/73/EC, B.4, "Acute Toxicity: Dermal Irritation/Corrosion" (2004); US EPA, OPPTS 870.2500, Acute Dermal Irritation (1998) and JMAFF, Japanese Test Guidelines (2000) including the most recent partial revisions.

Three rabbits were exposed to 0.5 ml of FR-1435X, applied onto clipped skin for 4 hours using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure.

Exposure to FR-1435X resulted in well-defined erythema and very slight oedema in the treated skin-areas of the three rabbits. The skin irritation had resolved within 48 hours after exposure in two animals and within 72 hours after exposure in the other animal.

Remnants of the test substance were present on the skin on day 1.

Treatment with FR-1435X resulted in mild reversible irritation of the skin on three of three animals. All indications of irritation had resolved within 72 hrs in all animals.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start : 09 November 2004 Completion : 26 November 2004

5.2. Aims of the study

The purpose of this primary skin irritation study was to assess the possible irritation or corrosion potential of a single dose of the test substance when administered to the intact skin of rabbits. This study should provide a rational basis for risk assessment in man.
The absence of skin pigmentation in the albino rabbit facilitates the evaluation of induced skin reactions. The dermal route was selected because the test substance may accidentally come into contact with the skin during manufacture, handling and/or use.

5.3. Guidelines

As required by the Dutch Act on Animal Experimentation (February 1997), the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-09). The study procedures described in this report were based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.404: "Acute Dermal Irritation / Corrosion", Paris Cedex, 2002.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 2004/73/EC, B.4: "Acute Toxicity: Dermal Irritation/Corrosion", 2004.

United States Environmental Protection Agency (EPA). Health Effects Test Guidelines, OPPTS 870.2500, Acute Dermal Irritation. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-98-196, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000, including the most recent partial revisions.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test Substance

The sponsor is responsible for all test substance data unless determined by NOTOX.

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)

6.1.2. Test substance preparation

The test substance was applied undiluted as delivered by the sponsor.

6.2. Test System

Species	Albino Rabbit, New Zealand White, (SPF-Quality) Recognised by international guidelines as the recommended test system (e.g. EC, OECD) Source: Charles River Deutschland, Kisslegg, Germany
Number of animals	3 Males.
Age and body weight	Animals used within the study were at least 6 weeks old and body weights were at least 1.0 kg.
Identification	Earmark.

6.3. Animal husbandry

Conditions
Animals were housed in a controlled environment, in which optimal conditions were considered to be approximately 15 air changes per hour, a temperature of 21.0 ± 3.0°C (actual range: 19.7 - 21.0°C), a relative humidity of 30-70% (actual range: 47 - 67%) and 12 hours artificial fluorescent light and 12 hours darkness per day.

Accommodation
Individually in labelled cages with perforated floors (Scanbur, Denmark, dimensions 56x44x37.5 cm).
Acclimatisation period was at least 5 days before start of treatment under laboratory conditions.

Diet
Standard laboratory rabbit diet (Charles River Breeding and Maintenance Diet for Rabbits, Altromin, Lage, Germany) approx. 100 g. per day. Certificates of analysis were examined and retained in the NOTOX archives. In addition, hay (BMI, Helmond, the Netherlands) was provided at least three times a week.

Water
Free access to tap-water. Certificates of quarterly analysis were examined and retained in the NOTOX archives.

Results of analysis for ingredients and/or contaminants of diet and water were assessed and did not reveal any findings that were considered to have affected study integrity.

6.4. Treatment

All available data relevant to the potential dermal irritation/corrosivity of the substance indicated that no severe effects were to be expected. An in-vitro test was considered, but a negative test result was anticipated that still would have to be confirmed in an in-vivo study. Since no severe harm for the animals was to be expected, this in-vivo skin irritation study was performed and was started by treatment of a single rabbit (sentinel). The two other animals were treated in a similar manner two weeks later, after considering the degree of skin irritation observed in the first animal.

Approximately 24 hours before treatment, the dorsal fur was clipped with electric clippers, exposing an area of approximately 150 square centimeters (10x15 cm^2). Whenever considered necessary the treated skin areas were re-clipped at least 3 hours before the observations, to facilitate scoring.

A health inspection was performed prior to the commencement of treatment, to ensure that the animals were in a good state of health. Special attention was paid to the skin to be treated, which was intact and free from abnormalities.

Each animal was treated by dermal application of 0.5 ml of the test substance. The test substance was applied to the skin of one flank, using a metalline patch* of 2x3 cm. The patch was mounted on Micropore tape*, which was wrapped around the abdomen and secured with Coban elastic bandage*.
Four hours after the application, the dressing was removed and the skin cleaned of residual test substance using water and watery ethanol (50% v/v).

*. Suppliers: Lohmann GmbH, Neuwied, Germany (Metalline) and 3M, St. Paul, Minnesota, U.S.A. (Micropore and Coban).

6.5. Observations

Mortality/Viability	Twice daily.
Toxicity	At least once daily.
Body Weight	Day of treatment (prior to application) and at termination.
Irritation	The skin reactions were assessed at approximately 1, 24, 48 and 72 hours after the removal of the dressings and test substance. The irritation scores and a description of all other (local) effects were recorded. Adjacent areas of the untreated skin of each animal served as controls.

The irritation was assessed according to the following numerical scoring system. At each observation, the highest scores given were recorded:

Erythema and eschar formation:

No erythema 0
Very slight erythema (barely perceptible) 1
Well-defined erythema 2
Moderate to severe erythema 3
Severe erythema (beet redness) * 4

*. Where signs of necrosis or corrosion (injuries in depth) prevent erythema scoring, the maximum grade for erythema (= 4) is given.

Oedema formation:

No oedema 0
Very slight oedema (barely perceptible) 1
Slight oedema (edges of area well-defined by definite raising) 2
Moderate oedema (raised approximately 1 millimeter) 3
Severe oedema (raised more than 1 millimeter and extending beyond the area of exposure) 4

6.6. Histopathology

No histopathology was performed.

6.7. Interpretation

No formal interpretation was done.

6.8. List of protocol deviations

There were no deviations from the protocol.

7. RESULTS

7.1. Irritation (Table 1)

Four hours exposure to 0.5 ml of FR-1435X resulted in well-defined erythema and very slight oedema in the treated skin-areas of the three rabbits.
The skin irritation had resolved within 48 hours after exposure in two animals and within 72 hours after exposure in the other animal.

7.2. Corrosion

There was no evidence of a corrosive effect on the skin.

7.3. Colouration / Remnants

No staining of the treated skin by the test substance was observed. Remnants of the test substance were present on the skin on day 1.

7.4. Toxicity / Mortality

No symptoms of systemic toxicity were observed in the animals during the test period and no mortality occurred.

8. CONCLUSION

Treatment with FR-1435X resulted in mild reversible irritation of the skin on three of three animals. All indications of irritation had resolved within 72 hrs in all animals.

Table 1: INDIVIDUAL SKIN IRRITATION SCORES

Following exposure, the treated skin-area remained sticky after removal of the test substance.

Animal #	327 (sentinel)			363			364		
Time after exposure	Erythema	Oedema	comments	Erythema	Oedema	comments	Erythema	Oedema	comments
1 hour	2	1	b	2	1	b	2	1	b
24 hours	1	0	-	1	0	-	1	0	-
48 hours	0	0	-	1	0	-	0	0	-
72 hours	0	0	-	0	0	-	0	0	-

Comments:
b. Dry remnants of the test substance present.

Table 2: MEAN VALUE IRRITATION SCORES

Animal #	Mean 24 – 72 hrs	
	Erythema	Oedema
327	0.3	0.0
363	0.7	0.0
364	0.3	0.0

Animal specifications:

Animal no	Sex	Age at start (weeks)	Body weights (grams) prior to application	at termination
327	♂	8-10	1959	2086
363	♂	8-10	1648	1779
364	♂	8-10	1572	1666

Appendix 1

GLP Certificate

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 8-12, 17 and 23 April, 1 and 3 May 2002 at

Notox
Safety & Environmental Research B.V.
Hambakenwetering 7, P.O. Box 3476
5203 DL 's Hertogenbosch

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Physical-chemical testing; Toxicity studies; Mutagenicity studies; Environmental toxicity studies on aquatic and terrestrial organisms; Studies on behaviour in water, soil, and air, bioaccumulation; Residue studies; Analytical and clinical chemistry testing; Toxicokinetic studies; In-vitro metabolism tests.

The H[illegible] September 2002

[illegible]elder, DVM
GLP Com[illegible] Department

Inspectorate for Health Protection and Veterinary Public Health
Ministry of Health, Welfare and Sport

Appendix 2

Certificate of Analysis

ICL Industrial Products

IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel:972-4-8469553 Fax:972-4-8469320 zilbermanj@tami-imi.icl-ip.com

Certificate of Analysis

FR-1435X

Brominated alkyl aryl compound

Batch No.	38278-53-4
Appearance, Color	Brown liquid
Assay, % (HPLC area)	Min. 97
Water, ppm	<500
Br total, %	63
Density, kg/m^3 at 25°C	~2000
Viscosity, cps at 23°C	1600-2000
Acidity, meq/g	<0.1
Solubility, g/100 g Water Toluene	 <0.1 complete

Appendix 3

Copy of protocol and amendments

ORIGINAL

PROTOCOL

Study Title

PRIMARY SKIN IRRITATION/CORROSION STUDY WITH FR-1435X IN THE RABBIT (4-HOUR SEMI-OCCLUSIVE APPLICATION)

Author

Drs. A.H.B.M. van Huygevoort

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

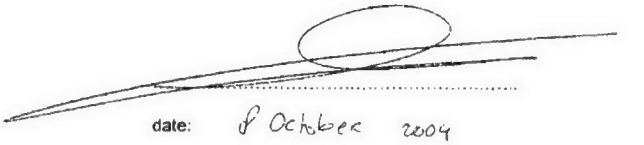
NOTOX Project 419873
NOTOX Substance 146232/A

1. CONTENTS

FR-1435X NOTOX project 419873

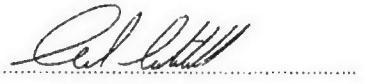
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Drs. A.H.B.M. van Huygevoort



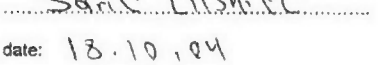
date: 8 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



date: 12/10/04

SPONSOR: Dead Sea bromine Group
Dr. S. Lifshitz



date: 18.10.04

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start week beginning :08 November 2004 (week 46) Completed week beginning :26 December 2004 (week 52) Proposed draft reporting date: 13 February 2005 (week 06)

3.2. Aims of study

The purpose of this primary skin irritation study is to assess the possible irritation or corrosion potential of a single dose of the test substance when administered to the intact skin of rabbits. This study should provide a rational basis for risk assessment in man.
The absence of skin pigmentation in the albino rabbit facilitates the evaluation of induced skin reactions. The dermal route is selected because the test substance may accidentally come into contact with the skin during manufacture, handling and/or use.

3.3. Guidelines

This protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-09) as required by the Dutch Act on Animal Experimentation (February 1997). The study procedures described in this protocol are based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.404: "Acute Dermal Irritation/ Corrosion", Paris Cedex, 2002.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 2004/73/EC, B.4: "Acute Toxicity - Skin Irritation", 2004.

United States Environmental Protection Agency (EPA). Health Effects Test Guidelines, OPPTS 870.2500, Acute Dermal Irritation. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-98-196, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000, including the most recent partial revisions.

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

The sponsor is responsible for all test substance data unless determined by NOTOX.
All available information on characteristics of the test substance will be taken into account before start of the study in order to determine if they conflict with/might affect the proceeding of the study.

CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	
Water	Not indicated
1% Aq. Carboxymethyl cellulose	Not indicated
Corn oil	Not indicated
Propylene glycol	Not indicated
Polyethylene glycol	Not indicated
Methyl ethyl ketone	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Olive oil	Not indicated
Dimethyl formamide	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

4.1.2. Test substance preparation

If a liquid	The test substance will be administered undiluted as delivered by the sponsor.
If a solid	If necessary, the test substance will be ground to a powder prior to weighing. The weighed samples will be applied after moistening with water (Milli-U), to ensure close contact to the skin. If the test substance is not miscible with water, watery ethanol or acetone (50% v/v) (in order of preference) will be used to moisten the test substance.

4.2. Test system

Species	Albino rabbit, New Zealand White, (SPF-Quality). Recognised by international guidelines as the recommended test system (e.g. EC, OECD) Based on availability one of the following sources will be used: - Charles River Deutschland, Kisslegg, Germany. - Charles River UK Limited, Margate, Kent, England. - Harlan, Horst, The Netherlands The source used will be specified in the raw data and report.
Number of animals	3 animals of one sex.
Age and body weight	Animals to be used within the study will be at least 6 weeks old and body weights will be at least 1.0 kg.
Identification	Earmark.

4.3. Animal husbandry

Conditions
A controlled environment is maintained in the room with optimal conditions of approximately 15 air changes per hour, a temperature of 21.0±3.0°C, a relative humidity of 30-70% and a 12 hour light/12 hour dark cycle. Temporary deviations from the maximum level for relative humidity (with a maximum of 20%) and light/dark cycle (with a maximum of 1 hour) may occur due to cleaning procedures or performance of functional observations in the room. Based on laboratory historical data these deviations are considered not to affect the study integrity.

Accommodation
Individually housed in labelled cages with perforated floors (Scanbur, Denmark, dimensions 56x44x37.5 cm). The acclimatisation period will be at least 5 days before the start of treatment under laboratory conditions.

Diet
Standard laboratory rabbit diet (Charles River Breeding and Maintenance Diet for Rabbits, Altromin, Lage, Germany) approx. 100 g. per day. Certificates of analysis are examined and then retained in the NOTOX archives. In addition, hay (BMI, Helmond, the Netherlands) will be provided at least three times weekly.

Water
Free access to tap-water. Certificates of quarterly analysis are examined and then retained in the NOTOX archives.

4.4. Treatment

All available data relevant to the potential dermal irritation/corrosivity of the substance will be taken into account prior to start of the test.

In principle, the study will be performed in a stepwise manner and will start with the treatment of one animal (sentinel). The criteria to treat additional animals are described in NOTOX Standard Operating Procedure DIE J/125. If no corrosion of the skin is observed the other animal(s) will be treated in a similar manner. If results suggest a corrosive effect, further testing in the other animal(s) will not be done.
If however no significant effects are to be expected, the study will start with the treatment of three rabbits, approved by the study director in the study files.

Approximately 24 hours before treatment, the dorsal fur will be clipped with an electric clipper, exposing an area of approximately 150 square centimetres (10x15 cm^2). To facilitate scoring, the treated skin-area may be re-clipped and/or cleaned with water or an appropriate solvent at least 3 hours before a next skin reading.

A health inspection will be performed prior to treatment, to ensure that the animals are in a good state of health. Special attention will be paid to the skin to be treated, which will be intact and free from any abnormality.

Each animal will be treated by dermal application of 0.5 ml (if a liquid) or 0.5 grams (if a solid) of the test substance to the intact, clipped skin of one flank using a Metalline patch# of 2x3 cm. The patch will be mounted on Micropore tape #, which will be wrapped around the abdomen and secured with Coban elastic bandage #.
Four hours after the application, the dressing will be removed and the skin cleaned of residual test substance using water and, if considered necessary, an appropriate solvent.
Suppliers: Lohmann GmbH, Neuwied, Germany (Metalline) and 3M, St. Paul, Minnesota, U.S.A (micropore and coban).

4.5. Observations

Mortality/Viability	At least twice daily. Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7).
Toxicity	If toxicity is observed, all clinical signs will be recorded at least once daily until they have disappeared.
Body Weight	On day 1 (prior to treatment) and at termination or at death (if found dead after day 1).
Necropsy	Animals found dead or animals sacrificed for humane reasons will be subjected to necropsy. Descriptions of all internal macroscopic abnormalities will be recorded. No necropsy will be performed on animals sacrificed for severe irritation/corrosion of the skin.
Irritation	The skin reactions will be assessed approximately 1, 24, 48, 72 hours after the removal of the dressing and test substance. At least for the duration of the skin reactions, further observations will be made 7 and 14 (maximum) days after exposure. The irritation scores and a description of all other (local) effects will be recorded. Adjacent areas of untreated skin of each animal serve as controls.

The irritation will be assessed according to the following numerical scoring system. At each observation, the highest scores given will be recorded:

Erythema and eschar formation:

No erythema 0
Very slight erythema (barely perceptible) 1
Well-defined erythema 2
Moderate to severe erythema 3
Severe erythema (beet redness) 4
*. Where signs of necrosis or corrosion (injuries in depth) prevent erythema scoring, the maximum grade for erythema (= 4) is given.

Oedema formation:

No oedema 0
Very slight oedema (barely perceptible) 1
Slight oedema (edges of area well-defined by definite raising) 2
Moderate oedema (raised approximately 1 millimetre) 3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure) 4

4.6. Histopathology

If skin reactions are masked by staining by the test substance, histological examination of the treated and control skin may be considered necessary. If so, the animals will be sacrificed immediately after the 72-hour observation and the skin samples will be collected and prepared for microscopy. The microscopical examination will be performed by a pathologist. Performance of this investigation will only be carried out after consultation with the sponsor and will be confirmed by an amendment.

4.7. Interpretation

The results will be evaluated according to the OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998) and the EC criteria for classification and labelling of dangerous substances and preparations (Council Directive 67/548/EEC and all adaptations to technical progress and amendments of this Directive published in the Official Journal of the European Communities).

5. DISTRIBUTION

Original: Study Director
1 Copy: Coordinating Biotechnician (via Study Director)
1 Copy: QAU/Management
1 Copy: Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO : 1

Study Title	Primary skin irritation/corrosion study with FR-1435x in the rabbit (4-hour semi-occlusive application)
Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A (file 146223)
NOTOX Project	419873

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1. Test substance information:

Description	Brown liquid

2. Page 4, paragraph 3.1 Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1.+ 2. The data has been updated according to information of the sponsor, dated January 06, 2005

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort date: 17 January 2005

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title	Primary skin irritation/corrosion study with Fr-1435x in the rabbit (4-hour semi-occlusive application)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419873

AMENDMENT DESCRIPTION

1. Page 6, paragraph 5.1 Test substance information:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

date: 28 February 2005

ORIGINAL

PROTOCOL AMENDMENT NO : 3

Study Title	Primary skin irritation/corrosion study with Fr-1435x in the rabbit (4-hour semi-occlusive application)
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419873

AMENDMENT DESCRIPTION

No formal interpretation will be done

REASONS FOR AMENDMENT

The sponsor requested this.

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

date: 26 May 2005

- Attachment 9 -

REPORT

Study Title

ACUTE EYE IRRITATION/CORROSION STUDY WITH FR-1435X IN THE RABBIT

Author

Drs. A.H.B.M. van Huygevoort

Study completion date

02 June 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419884
NOTOX Substance 146232/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

NOTOX B.V.

Drs. A.H.B.M. van Huygevoort
Study Director

Date: 2 June 2005

Drs. M.S. Teunissen
Section Head Toxicology

Date: 09 June 2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	protocol	12-Oct-04	12-Oct-04	12-Oct-04
	Report	04-Mar-05	04-Mar-05	07-Mar-05
Process	**SPF unit**	1-Nov-04	11-Nov-04	11-Nov-04
	Test substance handling			
	Exposure			
	Observation/Measurement			
	Specimen handling			

Head of Quality Assurance
C.J.Mitchell B.Sc.

i.A

Date: 13 jun 2005

4. SUMMARY

Acute eye irritation/corrosion study with FR-1435X in the rabbit.

The study was carried out based on the guidelines described in:
- OECD No.405 (2002) "Acute Eye Irritation / Corrosion"
- EC Commission Directive, Annex 5, B.5, (2004) "Acute Toxicity: Eye Irritation / Corrosion"
- EPA, OPPTS 870.2400 (1998): "Acute Eye Irritation"
- JMAFF guidelines (2000) including the most recent partial revisions.

Single samples of 0.1 ml of FR-1435X were instilled into one eye of each of three rabbits. Observations were made 1, 24, 48 and 72 hours after instillation.

Instillation of the test substance resulted in irritation of the conjunctivae, which consisted of redness and discharge. The irritation had completely resolved within 24 hours in all animals.

Treatment with FR-1435X caused mild, reversible, conjunctival irritation, without involvement of the cornea or iris, in the eye of three (3 / 3) rabbits.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine CompoundsLtd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start : 15 November 2004 Completion : 02 December 2004

5.2. Aims of the study

The purpose of this acute eye irritation/corrosion study was to assess the possible irritation or corrosion potential when a single dose of the test substance was placed in the conjunctival sac of the rabbit eye.
This study should provide a rational basis for risk assessment in man.
The absence of eye pigmentation in the albino rabbit facilitates the evaluation of induced eye reactions. The ocular route was selected because the test substance may accidentally come into contact with the eyes during manufacture, handling and/or use.

5.3. Guidelines

As required by the Dutch Act on Animal Experimentation (February 1997), the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-10). The study procedures described in this report were based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.405, "Acute Eye Irritation / Corrosion", Paris Cedex, 2002.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 2004/73/EC, B.5: "Acute Toxicity: Eye Irritation / Corrosion", 2004.

United States Environmental Protection Agency (EPA). Health Effects Test Guidelines, OPPTS 870.2400, Acute Eye Irritation. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-98-195, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000, including the most recent partial revisions.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test Substance

The sponsor is responsible for all test substance data unless determined by NOTOX.

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000

6.1.2. Test substance preparation

The test substance was instilled undiluted as delivered by the sponsor.

6.2. Test System

Species	Albino Rabbit, New Zealand White, (SPF-Quality) Recognised by international guidelines as the recommended test system (e.g. EC, OECD) Source: Charles River Deutschland, Kisslegg, Germany
Number of animals	3 Males.
Age and body weight	Animals used within the study were at least 6 weeks old and body weights were at least 1.0 kg.
Identification	Earmark.

6.3. Animal Husbandry

Conditions
Animals were housed in a controlled environment, in which optimal conditions were considered to be approximately 15 air changes per hour, a temperature of 21.0 ± 3.0°C (actual range: 19.8 - 21.3°C), a relative humidity of 30-70% (actual range: 47 - 67%) and 12 hours artificial fluorescent light and 12 hours darkness per day.

Accommodation
Individually in labelled cages with perforated floors (Scanbur, Denmark, dimensions 56x44x37.5 cm). Acclimatisation period was at least 5 days before start of treatment under laboratory conditions.

Diet
Standard laboratory rabbit diet (Charles River Breeding and Maintenance Diet for Rabbits, Altromin, Lage, Germany) approx. 100 g. per day. Certificates of analysis were examined and retained in the NOTOX archives. In addition, hay (BMI, Helmond, the Netherlands) was provided at least three times a week.

Water
Free access to tap-water. Certificates of quarterly analysis were examined and retained in the NOTOX archives.

Results of analysis for ingredients and/or contaminants of diet and water were assessed and did not reveal any findings that were considered to have affected study integrity.

6.4. Treatment

All available data relevant to the potential eye irritation/corrosivity of the substance indicated that no severe effects were to be expected. No severe reactions were noted in the skin irritation study (NOTOX Project 419873). An in-vitro test was considered, but a negative test result was anticipated that still would have to be confirmed in an in-vivo study. Since no severe harm for the animals was to be expected, this in-vivo eye irritation study was performed and was started by treatment of a single rabbit (sentinel). The two other animals were treated in a similar manner two weeks later, after considering the degree of eye irritation observed in the first animal.

A health inspection was performed prior to commencement of treatment, to ensure that the animals were in a good state of health. Special attention was paid to the eyes, which were free from any abnormality.

Each animal was treated by instillation of 0.1 ml of the test substance, in the conjunctival sac of one of the eyes after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about one second to prevent loss of the test substance. The other eye remained untreated and served as the reference control.

Immediately after the 24-hour observation, a solution of 2% fluorescein in water (adjusted to pH 7.0) was instilled into both eyes of each animal to quantitatively determine corneal epithelial damage. Any bright green stained area, indicating epithelial damage, was estimated as a percentage of the total corneal area.

6.5. Observations

Mortality/Viability	Twice daily.
Toxicity	At least once daily.
Body Weight	Day of treatment (prior to instillation) and at termination.
Irritation	The eyes of each animal were examined approximately 1, 24, 48 and 72 hours after instillation of the test substance. The irritation scores and a description of all other (local) effects were recorded.

The irritation was assessed according to the following numerical scoring system. At each observation, the highest scores given were recorded:

CORNEAL IRRITATION
Opacity: degree of density (area most dense taken for reading)
No ulceration or opacity (may include slight dulling of normal lustre) 0
Scattered or diffuse areas of opacity, details of iris clearly visible 1
Easily discernible translucent area, details of iris slightly obscured 2
Nacreous area, no details of iris visible, size of pupil barely discernible 3
Opaque cornea, iris not discernible through the opacity 4

Area of cornea involved:
No ulceration or opacity 0
One quarter or less but not zero 1
Greater than one quarter, but less than half 2
Greater than half, but less than three quarters 3
Greater than three quarters, up to whole area 4

IRIS
Normal 0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia, or injection, any of these or combination thereof, iris still reacting to light (sluggish reaction is positive) 1
No reaction to light, hemorrhage, gross destruction (any or all of these) 2

CONJUNCTIVAL IRRITATION
Redness (refers to palpebrae and sclera, excluding cornea and iris):
Blood vessels normal 0
Some blood vessels definitely hyperaemic (injected) 1
Diffuse, crimson color, individual vessels not easily discernible 2
Diffuse beefy red 3

Chemosis (refers to lids and/or nictitating membranes):
No swelling 0
Any swelling above normal (includes nictitating membranes) 1
Obvious swelling with partial eversion of lids 2
Swelling with lids about half closed 3
Swelling with lids more than half closed 4

Discharge:
No discharge (may include small amounts observed in inner canthus of normal animals) 0
Any amount different from normal and/or lacrimation 1
Discharge with moistening of the lids and hairs just adjacent to lids 2
Discharge with moistening of the lids and hairs (considerable area around the eye) 3

Where standard lighting was considered inadequate for observing minor effects, eye examinations were performed using an ophthalmic examination lamp.
In cases of equivocal results when comparing the treated and untreated eyes, the illustrated guide from the Consumer Product Safety Commission, Washington, D.C. 20207 was used for additional control purposes.

6.6. Interpretation

No formal interpretation was done.

6.7. List of protocol deviations

There were no deviations from the protocol.

7. RESULTS

7.1. Irritation (Table 1)

Instillation of approximately 0.1 ml of FR-1435X into one eye of each of three rabbits resulted in irritation of the conjunctivae, which consisted of redness and discharge. The irritation had completely resolved within 24 hours in all animals.

No iridial irritation or corneal opacity was observed, and treatment of the eyes with 2% fluorescein, 24 hours after test substance instillation revealed no corneal epithelial damage.

7.2. Corrosion

There was no evidence of ocular corrosion.

7.3. Colouration / Remnants

No staining of (peri) ocular tissues by the test substance was observed and no test substance remnants were seen.

7.4. Toxicity / Mortality

No symptoms of systemic toxicity were observed in the animals during the test period and no mortality occurred.

8. CONCLUSION

Treatment with FR-1435X caused mild, reversible, conjunctival irritation, without involvement of the cornea or iris, in the eye of three (3 / 3) rabbits.

Table 1: INDIVIDUAL EYE IRRITATION SCORES

	Cornea			Iris	Conjunctivae			
Time after dosing	Opacity	Area	Fluor area (%)		Redness	Chemosis	Discharge	Comments
♂ No 350# (Sentinel)								
1 hour	0	0		0	1	0	1	-
24 hours	0	0	0	0	0	0	0	-
48 hours	0	0		0	0	0	0	-
72 hours	0	0		0	0	0	0	-
♂ No 390#								
1 hour	0	0		0	1	0	1	-
24 hours	0	0	0	0	0	0	0	-
48 hours	0	0		0	0	0	0	-
72 hours	0	0		0	0	0	0	-
♂ No 393#								
1 hour	0	0		0	1	0	1	-
24 hours	0	0	0	0	0	0	0	-
48 hours	0	0		0	0	0	0	-
72 hours	0	0		0	0	0	0	-

Fluor area (%): green staining (percentage of total corneal area) after fluorescein treatment.

Table 2: MEAN VALUE EYE IRRITATION SCORES

Animal #	Mean 24 – 72 hours			
	Corneal opacity	Iris	Conjunctivae	
			Redness	Chemosis
350	0	0	0	0
390	0	0	0	0
393	0	0	0	0

Animal specifications:

Animal no	Sex	Age at start (weeks)	Body weights (grams)	
			prior to application	at termination
350	♂	8-10	2094	2210
390	♂	7-9	1352	1451
393	♂	7-9	1350	1472

Appendix 1

GLP Certificate

[redacted]

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 8-12, 17 and 23 April, 1 and 3 May 2002 at

Notox
Safety & Environmental Research B.V.
Hambakenwetering 7, P.O. Box 3476
5203 DL 's Hertogenbosch

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Physical-chemical testing; Toxicity studies; Mutagenicity studies; Environmental toxicity studies on aquatic and terrestrial organisms; Studies on behaviour in water, soil, and air, bioaccumulation; Residue studies; Analytical and clinical chemistry testing; Toxicokinetic studies; In-vitro metabolism tests.

The Hague, 18 September 2002

NEDERLAND

[redacted] Helder, DVM
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Ministry of Health, Welfare and Sport

Appendix 2

Certificate of Analysis

ICL Industrial Products

IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel:972-4-8469553 Fax:972-4-8469320 zilbermani@tami-imi.icl-ip.com

Certificate of Analysis

FR-1435X

Brominated alkyl aryl compound

Batch No.	38278-53-4
Appearance, Color	Brown liquid
Assay, % (HPLC area)	Min. 97
Water, ppm	<500
Br total, %	63
Density, kg/m^3 at 25°C	~2000
Viscosity, cps at 23°C	1600-2000
Acidity, meq/g	<0.1
Solubility, g/100 g Water Toluene	 <0.1 complete

Appendix 3

Copy of protocol and amendments

ORIGINAL

PROTOCOL

Study Title

ACUTE EYE IRRITATION/CORROSION STUDY WITH FR-1435X IN THE RABBIT

Author

Drs. A.H.B.M. van Huygevoort

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419884
NOTOX Substance 146232/A

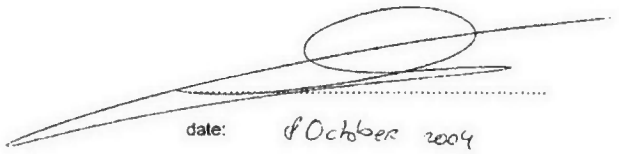
FR-1435X NOTOX Project 419884

1. CONTENTS

FR-1435X NOTOX Project 419884

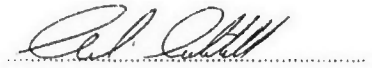
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Drs. A.H.B.M. van Huygevoort



date: 8 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



date: 12/10/04.

SPONSOR: Dead Sea bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

date: 18.10.04

FR-1435X NOTOX Project 419884

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start week beginning :15 November 2004 (week 47) Completed week beginning :02 January 2005 (week 53) Proposed draft reporting date: 13 February 2005 (week 06)

3.2. Aims of study

The purpose of this acute eye irritation study is to assess the possible irritation or corrosion potential to the rabbit eye following a single administration of the test substance.
This study should provide a rational basis for risk assessment in man.
The absence of eye pigmentation in the albino rabbit facilitates the evaluation of induced eye reactions. The ocular route is selected because the test substance may accidentally come into contact with the eyes during manufacture, handling and/or use.

3.3. Guidelines

This protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-10) as required by the Dutch Act on Animal Experimentation (February 1997). The study procedures described in this protocol are based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No. 405, "Acute Eye Irritation / Corrosion", Pans Cedex, 2002.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the determination of Toxicity, as last amended by Commission Directive 2004/73/EC, B.5: "Acute Toxicity - Eye Irritation", 2004.

United States Environmental Protection Agency (EPA). Health Effects Test Guidelines, OPPTS 870.2400, Acute Eye Irritation. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-98-195, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000; including the most recent partial revisions.

FR-1435X NOTOX Project 419884

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.
NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

FR-1435X NOTOX Project 419884

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

The sponsor is responsible for all test substance data unless determined by NOTOX.
All available information on characteristics of the test substance will be taken into account before start of the study in order to determine if they conflict with/might affect the proceeding of the study.

CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stablility in vehicle	
Water	Not indicated
1% Aq. Carboxymethyl cellulose	Not indicated
Corn oil	Not indicated
Propylene glycol	Not indicated
Polyethylene glycol	Not indicated
Methyl ethyl ketone	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Olive oil	Not indicated
Dimethyl formamide	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

FR-1435X NOTOX Project 419884

4.1.2. Test substance preparation

If a liquid	The test substance will be administered as delivered by the sponsor.
If a solid	If necessary, the test substance will be ground to a fine powder prior to administration.

4.2. Test system

Species	Albino rabbit, New Zealand White, (SPF-Quality). Recognised by international guidelines as the recommended test system (e.g. EC, OECD) Based on availability one of the following sources will be used: - Charles River Deutschland, Kisslegg, Germany. - Charles River UK Limited, Margate, Kent, England. - Harlan, Horst, The Netherlands The source used will be specified in the raw data and report.
Number of animals	3 animals of one sex.
Age and body weight	Animals to be used within the study will be at least 6 weeks old and body weights will be at least 1.0 kg.
Identification	Earmark.

4.3. Animal husbandry

Conditions
A controlled environment is maintained in the room with optimal conditions of approximately 15 air changes per hour, a temperature of 21.0±3.0°C, a relative humidity of 30-70% and a 12 hour light/12 hour dark cycle. Temporary deviations from the maximum level for relative humidity (with a maximum of 20%) and light/dark cycle (with a maximum of 1 hour) may occur due to cleaning procedures or performance of functional observations in the room. Based on laboratory historical data these deviations are considered not to affect the study integrity.

Accommodation
Individually housed in labelled cages with perforated floors (Scanbur, Denmark, dimensions 56x44x37.5 cm). The acclimatisation will be at least 5 days before the start of treatment under laboratory conditions.

Diet
Standard laboratory rabbit diet (Charles River Breeding and Maintenance Diet for Rabbits, Altromin, Lage, Germany) approx. 100 g. per day. Certificates of analysis are examined and then retained in the NOTOX archives. In addition, hay (BMI, Helmond, the Netherlands) will be provided at least three times weekly.

Water
Free access to tap-water. Certificates of quarterly analysis are examined and then retained in the NOTOX archives.

4.4. Treatment

All available data relevant to the potential eye irritation/corrosivity of the substance will be taken into account prior to start of the test.

If severe effects are to be expected or if results from a skin irritation study demonstrated definitive corrosion or severe skin irritancy of the test substance, no further testing for eye irritancy will be performed, it being presumed that it will produce similar severe effects on the eyes. This will be done in consultation with the sponsor and confirmed by amendment.

FR-1435X NOTOX Project 419884

The study will be performed in a stepwise manner and will start with the treatment of one animal (sentinel). The criteria to treat additional animals are described in NOTOX Standard Operating Procedure DIE J/125. If no severe effects on the eyes are observed in the sentinel, the other animal(s) will be treated in a similar manner. If results suggest severe eye irritation or a corrosive effect, further testing in the other animal(s) will not be done.

A health inspection will be performed prior to commencement of treatment, to ensure that the animals are in a good state of health. Special attention will be paid to the eyes, which will be free from any abnormality.

Each animal will be treated by instillation of a volume of 0.1 ml of the test substance* in the conjunctival sac of one of the eyes after gently pulling the lower lid away from the eyeball. The lids will then be gently held together for about one second to prevent loss of test substance. The non-treated eye will serve as the control.

*. If a solid, the weight will be recorded. A maximum of approximately 100 mg will be instilled.

Immediately after the 24 hour examination, both eyes of each animal will be further examined with the aid of fluorescein stain. Any bright green stained area, indicating epithelial damage, will be estimated as a percentage of the total corneal area. If epithelial damaging is observed this procedure will be repeated at later observation times, to assess recovery.

Immediately after completion of the 24 hour examination the test eye of each animal may be rinsed with tap water to remove residual test substance. For reference control, the other eye will also be rinsed.

4.5. Observations

Mortality/Viability	At least twice daily. Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7).
Toxicity	If toxicity is observed, all clinical signs will be recorded at least once daily until they have disappeared.
Body weight	On day 1 (prior to treatment) and at termination or at death (if found dead after day 1).
Necropsy	Animals found dead or animals sacrificed for humane reasons will be subjected to necropsy. Descriptions of all internal macroscopic abnormalities will be recorded. No necropsy will be performed on animals sacrificed for severe irritation/corrosion of the eye.
Irritation	The eyes of each animal will be examined approximately 1, 24, 48 and 72 hours after administration of the test substance. At least for the duration of the irritation (including local effects and staining of the eye), further observations will be made 7, 14 and 21 (maximum) days after administration. The irritation scores and a description of all other (local) effects will be recorded.

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The irritation will be assessed according to the following numerical scoring system. At each observation, the highest scores given will be recorded:

CORNEAL IRRITATION
Opacity: degree of density (area most dense taken for reading)
No ulceration or opacity (may include slight dulling of normal lustre).....0
Scattered or diffuse areas of opacity, details of iris clearly visible 1
Easily discernible translucent area, details of iris slightly obscured 2
Nacreous area, no details of iris visible, size of pupil barely discernible 3
Opeque cornea, iris not discernible through the opacity 4

Area of cornea involved:
No ulceration or opacity.....0
One quarter or less but not zero 1
Greater than one quarter, but less than half 2
Greater than half, but less than three quarters 3
Greater than three quarters, up to whole area 4

IRIS
Normal 0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia, or injection, any of these or combination thereof, iris still reacting to light (sluggish reaction is positive) 1
No reaction to light, hemorrhage, gross destruction (any or all of these) 2

CONJUNCTIVAL IRRITATION
Redness (refers to palpebrae and sclera, excluding cornea and iris):
Blood vessels normal 0
Some blood vessels definitely hyperaemic (injected) 1
Diffuse, crimson color, individual vessels not easily discernible 2
Diffuse beefy red 3

Chemosis (refers to lids and/or nictitating membranes):
No swelling 0
Any swelling above normal (includes nictitating membranes) 1
Obvious swelling with partial eversion of lids.....2
Swelling with lids about half closed 3
Swelling with lids more than half closed 4

Discharge:
No discharge (may include small amounts observed in inner canthus of normal animals) 0
Any amount different from normal and/or lacrimation 1
Discharge with moistening of the lids and hairs just adjacent to lids.....2
Discharge with moistening of the lids and hairs (considerable area around the eye) 3

Where standard lighting is considered inadequate for observing minor effects, eye examinations will be performed using an ophthalmic examination lamp.
In cases of equivocal results when comparing the treated and untreated eyes, the illustrated guide from the Consumer Product Safety Commission, Washington, D.C. 20207 will be used for additional control purposes.

4.6. Interpretation

The results will be evaluated according to the OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998) and the EC criteria for classification and labelling of dangerous substances and preparations (Council Directive 67/548/EEC and all adaptations to technical progress and amendments of this Directive published in the Official Journal of the European Communities).

5. DISTRIBUTION

Original: Study Director
1 Copy: Coordinating Biotechnician (via Study Director)
1 Copy: QAU/Management
1 Copy: Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO :1

Study Title	Acute eye irritation/corrosion study with FR-1435x in the rabbit
Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A (file 146223)
NOTOX Project	419884

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1. Test substance information:

Description	Brown liquid

2. Page 4, paragraph 3.1 Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1.+ 2. The data has been updated according to information of the sponsor, dated January 06, 2005

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

14 January 2005
date:

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title	Acute eye irritation/corrosion study with Fr-1435x in the rabbit
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419884

AMENDMENT DESCRIPTION

1. Page 6, paragraph.5.1 Tesl substance information:

Sponsor	Bromine Compounds Ltd P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

2 February 2005
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 3

Study Title	Acute eye irritation/corrosion study with Fr-1435X in the rabbit
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419884

AMENDMENT DESCRIPTION
No formal interpretation will be done.

REASONS FOR AMENDMENT

The sponsor requested this.

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

26 May 2005
date:

- Attachment 10 -

REPORT

Study Title

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR THE ANALYSIS OF FR-1435X IN ISO-MEDIUM

Author

Dr. Ir. C. Brands

Study completion date

February 18, 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Sponsor

Bromine Compounds Ltd.
P.O. Box 180
BEER-SHEVA 84101
Israel

Laboratory Project Identification

NOTOX Project 419838
NOTOX Substance 146232/A

1. CONTENTS

FIGURES

APPENDICES

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Dr. Ir. C. Brands
Study Director

Date: February 18, 2005

Dr. Ir. E. Baltussen
Section Head
Analytical & Physical Chemistry

Date: February 21, 2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase / Section	Start inspection date(s)	End Inspection date(s)	Reporting date
Protocol (Study)		05\OCT\04	05\OCT\04	05\OCT\04
On-site (Process)	Analytical & Physical Chemistry	04\OCT\04	12\OCT\04	25\OCT\04
Report (Study)		29\NOV\04	29\NOV\04	29\NOV\04

Head of Quality Assurance
C.J.Mitchell B.Sc.
pp

J.T.G. Wiltschet

Date: February 22, 2005

4. SUMMARY

A high performance liquid chromatographic (HPLC) method for quantitative analysis of the test substance (FR-1435X) in ISO-medium was implemented. A Zorbax RX-C18 column was used with 90/10 (v/v) Methanol/Milli-Q water as the mobile phase and a spectrophotometric detector set to read the absorbance at 225 nm.

Standard solutions of the test substance were prepared in Methanol at exactly known concentrations between 860 mg/l and 1082 mg/l, ultrasonicated for at least 15 minutes to dissolve the test substance completely and diluted with Methanol. Calibration solutions for the validation tests were obtained by further diluting these dilutions in a 1:1 (v:v) ratio with ISO-medium. End solution for all samples was 50/50 (v/v) Methanol/ISO-medium. Accuracy samples were prepared using a 986 or 1016 mg/l test substance solution in Methanol.

The method was validated for the following parameters:

4.1. Specificity

Two test substance peaks were observed in chromatograms of test substance solutions. It was assumed that these peaks derive from the major components in the test substance. The area of the sum of the test substance peak was used as test substance response in the calculations during the validation tests. The chromatogram of a blank solution showed no peak with the same retention time as that of the major components of the test substance.

Since no interferences were detected in blank chromatograms, the specificity requirements were met and the analytical method was found to be specific for the test substance.

4.2. Linearity

A linear relationship between response and test substance concentration was found over a concentration range of 0.00601 – 0.601 mg/l (in end solution) using a ($1/concentration^2$) weighting factor. Since the correlation coefficient (r) of the weighted regression is > 0.990, the calibration line was accepted.

4.3. Accuracy

A blank solution (ISO-medium; 10 ml) was spiked in five-fold at concentration levels of 0.0251, 0.503, 0.996 and 5.03 mg/l. The 5.03 mg/l accuracy samples were ultrasonicated for 5 minutes. To all aqueous samples, 10 ml Methanol was added. In this way, a dilution factor of 2 was obtained. The 5.03 mg/l accuracy samples were further diluted by a factor of 10 with 50/50 (v/v) Methanol/ ISO-medium, resulting in a dilution factor of 20. At all concentration levels, mean recoveries between 70% and 110% were obtained. Therefore, the analytical method was considered applicable to samples in ISO-medium in the concentration range 0.0251 mg/l to 5.03 mg/l.

4.4. Repeatability

Based on the results from the accuracy samples, it was concluded that at all concentration levels the coefficient of variation was < 20%. Therefore, the method was found to be applicable for analysis of samples in ISO-medium in the concentration range of 0.0251 – 5.03 mg/l.

4.5. Limit of Quantification (LOQ)

Based on the results obtained during this project, the LOQ was determined to be 0.0251 mg/l in ISO-medium.

4.6. Limit of Detection (LOD)

The limit of detection for the test substance was determined to be 0.0021 mg/l (in end solution) at an injection volume of 100 µl. Taking a dilution factor of 2 into account, this corresponds to a limit of detection of 0.0042 mg/l in ISO-medium.

4.7. Stability of the Chromatographic System and End Solutions

The chromatographic system and end solutions containing the test substance 0.00601 and 0.601 mg/l) were stable for at least 15.5 hours when stored at room temperature in the dark.

4.8. Stability of Standard Solutions

Standard solutions (860 mg/l and 1040 mg/l) of the test substance in Methanol were stable for at least 168 hours when stored at room temperature.

4.9. Storage Stability of Samples

The test substance was found to be stable in ISO-medium in the concentration range 0.0254 to 5.08 mg/l during storage in the freezer for 18 hours.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Dr. Ir. C. Brands
Study Plan	Start : 22 October 2004 Completion : 13 November 2004

5.2. Aims of the study

The purpose of the study was to develop and validate a method for the quantitative analysis of FR-1435X in ISO-medium. A high performance liquid chromatographic (HPLC) method was implemented. The results of the method validation are summarised in this report.

5.3. Guidelines

The study procedure described in this report was based on the following guideline:

European Commission: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (Part A, section 4) and Annex III (Part A, section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

CONFIDENTIAL

Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)

6.2. Reagents

Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore Corp., Bedford, MA, USA
Methanol	HPLC-grade, Labscan, Dublin, Ireland
ISO-medium	Medium formulated using Milli-Ro water (tap water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$CaCl_2.2H_2O$	293.8 mg/l
$MgSO_4.7H_2O$	123.3 mg/l
$NaHCO_3$	64.8 mg/l
KCl	5.8 mg/l

6.3. Analytical method

6.3.1. Chromatography

A high performance liquid chromatographic (HPLC) method for quantitative analysis of the test substance, by analysis of the major components, was implemented by NOTOX B.V. The conditions are described below:

Column	
Stationary phase	Zorbax RX-C18
Dimensions	250 x 4.6 mm; dp = 5 µm
Brand	Agilent, Palo Alto, CA, USA
Mobile phase	90/10 (v/v) Methanol/Milli-Q water
Flow	1.2 ml/min
Detection	Spectrophotometric (wavelength of 225 nm)
Injection volume	100 µl

6.3.2. Preparation of Solutions

Standard solutions of the test substance were prepared in Methanol at exactly known concentrations between 860 mg/l and 1082 mg/l, ultrasonicated for at least 15 minutes to dissolve the test substance completely and diluted with Methanol. Calibration solutions for the validation tests were obtained by further diluting these dilutions in a 1:1 (v:v) ratio with ISO-medium. End solution for all samples was 50/50 (v/v) Methanol/ISO-medium.

Accuracy samples were prepared using a 986 mg/l or a 1016 mg/l test substance solution in Methanol or dilutions thereof in Methanol.

6.3.3. Data Acquisition and Processing

Data acquisition and data processing was performed using Empower (Waters, Milford, MA, USA) version 5.00.

6.4. Interpretation

Response: Peak area of test substance Peak 1 and Peak 2 [units]

Response factor: $Rf = \frac{Response}{c}$ [units * l/mg]

where:
c : concentration of test substance [mg/l]

Mean: $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$

where :
x_i : measured value
n : number of measurements

Standard deviation: $s_{n-1} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$

Coefficient of variation: $\frac{s_{n-1}}{\bar{x}} * 100\%$

Linearity: A linear regression program was used to calculate the regression line from the responses and concentrations. Linear regression analysis was performed using the least squares method. A (1/concentration^2) weighting factor was used.

Regression line: $Y = a * X + b$

where:
Y : response
X : concentration
a : slope
b : intercept

Correlation coefficient

During regression analysis, the correlation coefficient (r) was calculated.

Limit of detection

The limit of detection is defined as the lowest concentration of test substance that can be distinguished from instrumental noise using the analytical method described.

$$\text{Limit of detection} = \frac{3*N}{S} * C$$

where:
N : noise height [μAU]
S : test substance peak height [μAU]
C : concentration of test substance [mg/l]

Concentration analysed

$$\frac{(R-b)*d}{a} \text{ [mg/l]}$$

where:
R : response sample [units]
a : slope [units*l/mg]
b : intercept [units]
d : dilution factor

Recovery

$$\frac{\text{concentration analysed}}{\text{concentration prepared}} * 100\%$$

7. VALIDATION RESULTS

The analytical method specified under '6.3 Analytical method' was validated. The results are described below.

All calculations were performed using non-rounded values. Rounded values are reported in the tables. Therefore, some differences might be observed when recalculating the parameters as mentioned in the tables.

7.1. Specificity

HPLC chromatograms of a test substance and of a blank solution (50/50 (v/v) Methanol/ISO-medium) are shown in Figure 1 and Figure 2, respectively. Two test substance peaks were observed in chromatograms of test substance solutions. It was assumed that these peaks (Peak 1 and Peak 2) derive from the major components in the test substance. The area of the sum of the test substance peaks was used as test substance response in the calculations during the validation tests. The chromatogram of a blank solution showed no peak with the same retention time as that of the major components of the test substance.

Since no interferences were detected in blank chromatograms, the specificity requirements were met and the analytical method was found to be specific for the test substance.

7.2. Linearity

From two standard solutions, five dilutions were prepared. This resulted in a concentration range of 0.00601 – 0.601 mg/l (in end solution). Each of these calibration solutions was injected in duplicate, resulting in ten calibration points. Using a regression program, a calibration curve was fitted through the calibration points. Figure 3 illustrates the obtained calibration curve and Table 1 shows the corresponding statistical parameters.

Table 1 Linearity

Parameter	Value
Slope	$4.54*10^5$
Intercept with Y-axis	$3.59*10^2$
Weighting factor	$1/concentration^2$
r	0.9993

The calibration line was constructed using all data points. Each of the individual calibration points deviated less than 10% from the constructed calibration line.

From the results, it was concluded that there is a linear relationship between response and concentration in the concentration range of 0.00601 – 0.601 mg/l (in end solution) using a ($1/concentration^2$) weighting factor. Additionally, since the correlation coefficient (r) of the weighted regression was > 0.990, the calibration line was accepted.

7.3. Accuracy

A blank solution (ISO-medium; 10 ml) was spiked in five-fold (five samples at each concentration level) at concentration levels of 0.0251, 0.503, 0.996 and 5.03 mg/l using a 4.93, 98.6 (twice) or 986 mg/l test substance solution in methanol, respectively. The 5.03 mg/l accuracy samples were ultrasonicated for 5 minutes. To all aqueous samples, 10 ml Methanol was added. In this way, a dilution factor of 2 was obtained. The 5.03 mg/l accuracy samples were further diluted by a factor of 10 with 50/50 (v/v) Methanol/ISO-medium, resulting in a dilution factor of 20. All samples were analysed by single injection into the analytical system. The results are summarised in Table 2.

Table 2 Results of the accuracy samples

Concentration prepared [mg/l] [1]	Area	Concentration analysed [mg/l] [2]	Recovery [%]	Mean Recovery [%]	Coefficient of variation [%]
0.0251	6196	0.0257	102		
0.0251	6278	0.0261	104		
0.0251	6049	0.0250	100	100	3.5
0.0251	6015	0.0249	99		
0.0251	5765	0.0238	95		
0.503	112256	0.493	98		
0.503	112348	0.493	98		
0.503	112939	0.496	99	100	2.2
0.503	117960	0.518	103		
0.503	115393	0.506	101		
0.996	214281	0.942	95		
0.996	211599	0.930	93		
0.996	219100	0.963	97	95	1.4
0.996	213028	0.936	94		
0.996	217095	0.954	96		
5.03	92344	4.05	81		
5.03	76556	3.35	67		
5.03	101782	4.47	89	81	12.2
5.03	89246	3.91	78		
5.03	105138	4.61	92		

[1] Concentration in ISO-medium not corrected for the spiking volume

[2] Taking a dilution factor of 2 or 20 into account

At all concentration levels, mean recoveries between 70% and 110% were obtained. Therefore, the analytical method was considered applicable to samples in ISO-medium in the concentration range 0.0251 to 5.03 mg/l.

7.4. Repeatability

Based on the results from the accuracy samples listed in Table 2, it was concluded that at all concentration levels the coefficient of variation was < 20%. Therefore, the method was found to be applicable for analysis of samples in ISO-medium in the concentration range of 0.0251 to 5.03 mg/l. The relatively high coefficient of variation for the 5.03 mg/l accuracy samples is probably due to the low solubility of the test substance in ISO-medium.

7.5. Limit of Quantification (LOQ)

The LOQ is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of less than 20% is demonstrated. Based on the results listed in Table 2, the LOQ is reported here as 0.0251 mg/l in ISO-medium.

7.6. Limit of Detection (LOD)

From a chromatogram of a 0.00400 mg/l solution of the test substance in end solution, the noise level (N) was determined to be 10.0 µAU. From the same chromatogram, the test substance signal (S), i.e. the height of the smallest test substance peak (Peak 2), was determined to be 57.4 µAU. Using these values, the limit of detection (S/N=3) was calculated to be 0.0021 mg/l (in end solution) at an injection volume of 100 µl. Taking a dilution factor of 2 into account, this corresponds to a limit of detection of 0.0042 mg/l in ISO-medium.

7.7. Stability of the Chromatographic System and End Solutions

A 0.00601 mg/l and a 0.601 mg/l solution of the test substance in end solution were injected seven times throughout the validation sequence (including the beginning and end). The results are summarised in Table 3.

Table 3 Stability of the chromatographic system and end solutions

Elapsed time (hours)	Concentration [mg/l]	Coefficient of variation of the response (n=7) [%]
15.7	0.00601	3.6
15.5	0.601	0.82

Since the coefficient of variation at both concentration levels was less than 20%, it was concluded that the chromatographic system was stable over at least a 15.5-hour time interval at the concentration levels tested.

7.8. Stability of Standard Solutions

Two standard solutions (860 and 1040 mg/l) in Methanol were stored at room temperature and measured 168 hours after preparation together with two freshly prepared standard solutions (986 and 1082 mg/l). Prior to measurement, each standard solution was diluted to an exactly known concentration of approximately 0.60 mg/l and analysed by single injection. The coefficient of variation of the response factor was 3.9%. Since this coefficient of variation is < 10%, it was concluded that the stored standard solutions were stable during storage.

A stronger criterion is used here than for the determination of the stability of the chromatographic system and end solutions. This is because the upper part of the calibrated range is used for determination of the stability of standard solutions. Standard deviations usually are lower in this range than in the lower part of the calibrated range.

7.9. Storage Stability of Samples

The storage stability of samples in ISO-medium was determined using two additional accuracy samples prepared identically and on each of the concentration levels as those listed in Table 2. These accuracy samples were stored in the freezer for 18 hours. After storage, the samples were defrosted at room temperature and ultrasonicated for 5 minutes, prior to treatment (i.e. addition of methanol and subsequent dilution if necessary) and analysis. The results are summarised in Table 4.

Table 4 Results of the storage stability samples

Concentration prepared [mg/l] [1]	Area	Concentration analysed [mg/l] [2]	Recovery [%]	Mean Recovery [%]
0.0254	6158	0.0255	101	99
0.0254	5971	0.0247	97	
0.498	105689	0.464	93	93
0.498	104455	0.458	92	
1.00	207287	0.911	91	92
1.00	211153	0.928	93	
5.08	104675	4.59	90	89
5.08	102165	4.48	88	

[1] Concentration in ISO-medium

[2] Taking a dilution factor of 2 or 20 into account

Since the recovery of the frozen accuracy samples deviated by less than 10% from the recovery of samples that were not frozen (see Table 2), the test substance was found to be stable in ISO-medium in the concentration range 0.0254 to 5.08 mg/l during storage in the freezer for 18 hours.

FIGURES

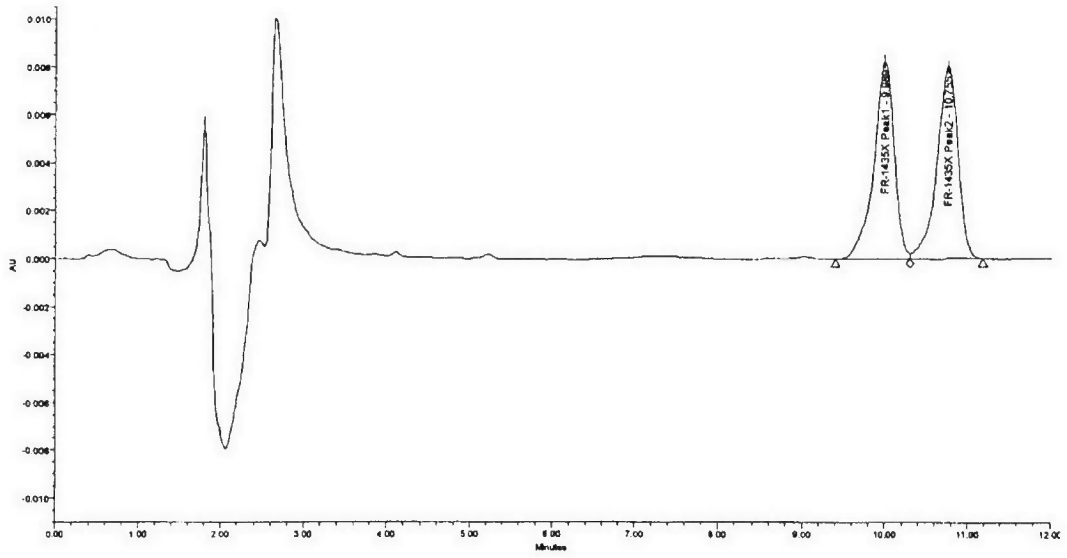

Figure 1 HPLC chromatogram of a 0.601 mg/l solution of FR-1435X in 50/50 (v/v) Methanol/ISO-medium [res.id. 3034]

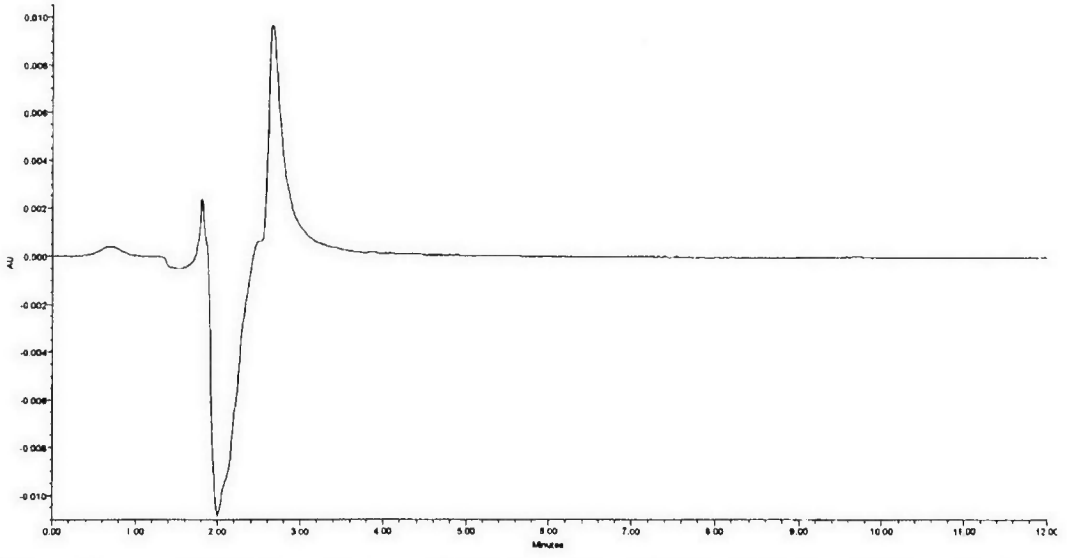

Figure 2 HPLC chromatogram of a blank solution (50/50 (v/v) Methanol/ISO-medium) [res.id. 3307]

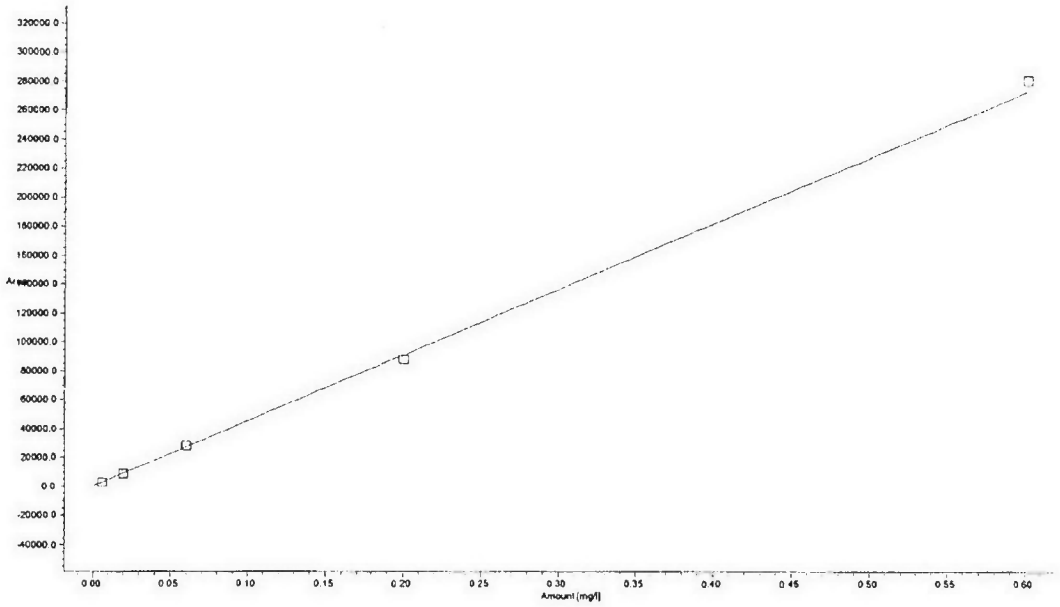

Figure 3 Regression line: response of the sum of FR-1435X Peak 1 and Peak 2 as a function of concentration [cal.curve id. 3032]

APPENDIX I COPY OF PROTOCOL & AMENDMENTS (12 PAGES)

ORIGINAL

PROTOCOL

Study Title

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR THE ANALYSIS OF FR-1435X IN ISO-MEDIUM

Author

Dr. Ir. C. Brands

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419838
NOTOX Substance 146232/A

- Page 1 of 8 -

FR-1435X NOTOX Project 419838

1. CONTENTS

FR-1435X NOTOX Project 419838

2. PROTOCOL APPROVAL

STUDY DIRECTOR: Dr. Ir. C. Brands

C Brands

date: 5 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.

date: 5-10-04.

SPONSOR: Dead Sea bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

date: 17.10.04

FR-1435X NOTOX Project 419838

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Dr. Ir. C. Brands
Study Plan	Start week beginning : 18 October 2004 (week 43) Completed week beginning : 08 November 2004 (week 46) Proposed draft reporting date : 05 December 2004

3.2. Aims of study

The purpose of this study is to develop and to validate a method for the quantitative analysis of the test substance in ISO-Medium. The method will be validated as described under "validation of the analytical method".

3.3. Guidelines

The study procedures described in this protocol are based on the following guideline:

European Commission: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (Part A, section 4) and Annex III (Part A, section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

FR-1435X NOTOX Project 419838

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

4. MATERIALS AND METHODS

4.1. Test substance

CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

The sponsor is responsible for all test substance data unless determined by NOTOX.

FR-1435X NOTOX Project 419838

4.2. Development of the analytical method

4.2.1. Chromatography

If the sponsor supplies an analytical method, it will be implemented (if possible) or a method based on that of the sponsor will be developed. If no analytical method is supplied, NOTOX will develop a High Performance Liquid Chromatographic (HPLC) or a Gas Chromatographic (GC) method. A deviating technique will not be used without consultation of the sponsor.

Method development work will not be reported, only the conditions of the final (validated) method will be presented in the report. When considered relevant, alternative matrices may also be tested for method performance (will also not be reported).

4.2.2. Preparation of Solutions

Solutions of the test substance and if applicable of an appropriate internal standard will be prepared in a suitable solvent.

4.3. Validation of the analytical method

Validation will be performed according to NOTOX SOP CHE/A/037 in case analysis is performed by HPLC or according to NOTOX SOP CHE/A/038 in case analysis is performed by GC. If an alternative technique is used, alternative procedures may apply.

The method will be validated for the following parameters. Under special conditions, different criteria might be considered acceptable.

4.3.1. Specificity

Chromatograms of the test substance will be compared with those of a blank. Interferences in blank chromatograms are required to be < 30% of the limit of quantification. Representative chromatograms of a blank and of a test substance solution will be shown in the report.

4.3.2. Linearity

From two standard solutions at least three dilutions will be prepared. Each of the solutions will be injected in duplicate. The responses will be plotted against concentrations, resulting in a regression line. A ($1/concentration^2$) weighting factor will be applied. The correlation coefficient is required to be at least 0.99 and the back calculated concentration of each individual calibration point must be within 90 –110% of the nominal concentration. If the back calculated concentration exceeds this range, the calibration point will be removed and the calculation is repeated. The number of accepted points should be at least 75% of the used calibration points with a minimum of 5. The obtained calibration curve will be shown in the report.

4.3.3. Accuracy

Blank matrix will be spiked in at least five-fold at a minimum of two concentration levels using either a concentrated test substance solution or pure test substance. The maximum concentration of organic solvent that will be used is 5%. For each spiked sample the recovery will be calculated. The mean recovery at each concentration level is required to be in the range of 70-110%. If required, outliers may be discarded as long as at least 75% of the prepared accuracy samples are accepted with a minimum of 4.

FR-1435X NOTOX Project 419838

4.3.4. Repeatability

The repeatability of the method will be determined from the results of the accuracy samples. The coefficient of variation at both concentration levels is required to be less than 20%.

4.3.5. Limit of Quantification (LOQ)

The limit of quantification will be determined from the results of the accuracy samples. The LOQ is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of less than 20% is demonstrated.

4.3.6. Limit of Detection (LOD)

A standard solution will be diluted to a sufficiently low concentration. The test substance peak height will be measured as well as the noise level of the system. If in chromatograms of a blank a peak at the test substance position is observed, a LOD cannot be determined.

4.3.7. Stability of the Chromatographic System and End Solutions

Two test substance solutions, preferably at the highest and lowest level of the calibration curve, will each be injected at least three times over a relevant time period. The coefficient of variation of the response at both concentration levels is required to be less than 20%.

4.3.8. Stability of standard solutions

Two standard solutions will be stored at room temperature and measured at least 12 hours after preparation, together with two freshly prepared standard solutions. Prior to measurement, each of the standard solutions will be diluted to a concentration level within the calibrated range. Since the upper part of the calibrated range is preferably used for determination of the stability of standard solutions, the coefficient of variation of the response factors is required to be < 10%.

4.3.9. Storage Stability of Samples

When relevant for further analysis, two additional accuracy samples at each concentration level can be prepared which are stored in a freezer prior to analysis. If the maan recovery of these samples deviates less than 10% from the recovery of the samples that were not frozen and falls in the range 70-110%, samples of the test substance are considered stable during freezing.

4.4. Interpretation

Response	Peak area or peak height (for GC/HPLC analysis)
Response factor	response / concentration
Mean:	$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} xi$

where :
xi : measured value
n : number of measurements

FR-1435X NOTOX Project 419838

Standard deviation	$Sn-1=\sqrt{\frac{\sum_{i=1}^{n}(xi-\bar{x})^2}{(n-1)}}$
Coefficient of variation	$\frac{Sn-1}{\bar{x}} * 100\%$
Linearity	Linear regression analysis will be performed using the least squares method.
Correlation coefficient (r)	During regression analysis, the correlation coefficient (r) will be calculated.
Limit of detection	The limit of detection is defined as the concentration of test substance at which the peak height is three times the noise level. Based on the noise and signal measurement, the limit of detection will be calculated.
Concentration analysed	Concentration measured in the accuracy samples.
Recovery	$\frac{\text{concentration analysed}}{\text{concentration prepared}} * 100\%$

5. DISTRIBUTION

Original: Study Director
1 Copy: Technical Coordinator
1 Copy: QAU/Management
1 Copy: Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO : 01

Study Title	Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419838

AMENDMENT DESCRIPTION

1. Sponsor — Dead Sea Bromine Group
Purchasing Division of DSBG
P.O. Box 180
BEER-SHEVA 84101
Israel

REASONS FOR AMENDMENT

1. The data has been updated because of a typing error.

APPROVAL
Study director

C Brands ..... October 14, 2003 .....

Dr. Ir. C. Brands — date:

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 02

Study Title	Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419838

AMENDMENT DESCRIPTION

1. Additional accuracy samples will be stored refrigerated, prior to analysis.

REASONS FOR AMENDMENT

1. In addition to the protocol, stability of refrigerated samples will be studied.

APPROVAL
Study director

C Brands ..... November 1, 2004 .....
Dr. Ir. C. Brands date:

Sponsor

Sarit Lifshitz ..... 7/11/04 .....
Dr. S. Lifshitz date:
Dead Sea Bromine Group

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 3

Study Title	Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419838

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

2. Page 5, paragraph 4.1. Test substance information:

Description	Brown liquid

REASONS FOR AMENDMENT

1.-2. The data has been updated according to information of the sponsor, dated January 06, 2005.

APPROVAL
Study director

C Brands
Dr. Ir. C. Brands

January 13, 2005
date

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 4

Study Title	Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419838

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005.

APPROVAL
Study director

C Brands

.....
Dr. Ir. C. Brands

February 18, 2005

.....
date:

Page 1 of 1

- Attachment 11 -

SafePharm Laboratories

FR-1435P:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SPL PROJECT NUMBER: 0466/0301

AUTHOR: A Sanders

STUDY SPONSOR:

Bromine Compounds Ltd.
Makleff House
P.O.B. 180
BEER-SHEVA 84101
ISRAEL

TEST FACILITY:

Safepharm Laboratories Limited
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	23 April 2007	Standard Test Method Compliance Audit
	17 January 2008	Test Material Preparation
	08 January 2008	Test System Preparation
	02 January 2008	Animal Preparation
	17 January 2008	Dosing
	02, 22 January 2008	Assessment of Response
§	06 March 2008	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

G Wren ..... DATE: 06 JUN 2008

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA ACQI FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: 30 MAY 2008

A Sanders
Study Director

CONTENTS

FR-1435P:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC
- United States Environmental Protection Agency Health Effects Test Guidelines OPPTS 870.2600 Skin Sensitisation March 2003

Methods. Following a preliminary screening test in which clinical signs of toxicity were noted at concentrations above 25% v/v, this concentration was therefore selected as the highest investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the test material as a solution in acetone/olive oil 4:1 at concentrations of 25%, 10% or 5% v/v. A further group of five animals was treated with acetone/olive oil 4:1 alone. A concurrent positive control test, using a group of five animals, was performed with the known sensitiser, α-Hexylcinnamaldehyde Tech 85%, at a concentration of 15% v/v in acetone/olive oil 4:1.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Treatment Group	Concentration (%v/v) in acetone/olive oil 4:1	Stimulation Index	Result
Test Material	5	1.34	Negative
	10	1.96	Negative
	25	3.14	Positive
Positive Control Material	15	5.05	Positive

The concentration of test material expected to cause a 3 fold increase in ^{3}HTdR incorporation (EC_3 value) was calculated to be 23.2% v/v in acetone/olive oil 4:1.

Conclusion. The test material was considered to be a sensitiser under the conditions of the test.

FR-1435P:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)

- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC

- United States Environmental Protection Agency Health Effects Test Guidelines OPPTS 870.2600 Skin Sensitisation March 2003

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test materials that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test material to man.

The study was performed between 07 January 2008 and 07 February 2008.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	FR-1435P
Description	:	amber coloured slightly viscous liquid
Batch number	:	38250-29-6
Date received	:	27 November 2007
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

A Certificate of Analysis supplied by the Sponsor is given in Appendix 5.

2.2 Preparation of Test Material

For the purpose of the study, the test material was used undiluted and also freshly prepared in acetone/olive oil 4:1. This vehicle was chosen as it produced the most suitable formulation at the required concentration. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

Determination, by analysis, of the concentration, homogeneity and stability of the test material preparations was not appropriate because it was not specified in the Study Plan and is not a requirement of the Test Guideline.

3. METHODS

3.1 Animals and Animal Husbandry

One female CBA/Ca (CBA/Ca CruBR) strain mouse was supplied by Charles River UK Limited, Margate, Kent, UK and twenty-seven female CBA/Ca (CBA/CaOlaHsd) strain mice were supplied by Harlan UK Limited, Bicester, Oxon, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (Certified Rat and Mouse Diet) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

Animals in which any adverse effects were noted that were considered to approach the moderate severity limit set forth in the UK Home Office Project Licence, were humanely killed.

3.2.1 Preliminary Screening Test

Using available information regarding the systemic toxicity/irritancy potential of the test material, a preliminary screening test was performed using three mice, one per test material concentration. The mice were treated by daily application of 25 µl of the undiluted test material or the test material at concentrations of 50% or 25% v/v in acetone/olive oil 4:1, to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mice were observed twice daily on Days 1, 2 and 3, the surviving mice once daily on Day 4 and the surviving mouse once daily on Days 5 and 6. Any signs of toxicity or excessive local irritation noted during this period were recorded. The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and of the surviving mouse on Day 6. The bodyweight of the mice that were humanely killed was recorded immediately prior to termination.

3.2.2 Main Test

3.2.2.1 Test Material Administration

Groups of five mice were treated with the test material at concentrations of 25%, 10% or 5% v/v in acetone/olive oil 4:1. The preliminary screening test suggested that the test material would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test

material formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of five mice received the vehicle alone in the same manner.

The positive control animals were similarly treated to the test animals except that 25 µl of the positive control material, α-Hexylcinnamaldehyde, at a concentration of 15% v/v in acetone/olive oil 4:1 was applied to the dorsal surface of each ear.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test material, vehicle control or positive control material (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml, specific activity 2.0 Ci/mmol, GE Healthcare UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube. The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4°C, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by β-scintillation counting. The "Poly Q^{TM}" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons were performed between control and treated groups. For homogenous datasets Dunnett's Multiple Comparison test was used and for non-homogenous datasets Dunnett's T3 Multiple Comparison Method was used.

Probability values (p) are presented as follows:

$P<0.001$	***
$P<0.01$	**
$P<0.05$	*
$P\geq0.05$	(not significant)

3.4 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph nodes from each individual animal and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test material or positive control material will be regarded as a sensitiser if at least one concentration of the test material or positive control material results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test material failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

At the request of study sponsor the EC_3 value was calculated. The EC_3 value is the concentration of test material expected to cause a 3 fold increase in ^{3}HTdR incorporation. The equation used for the calculation of EC_3 is:

$EC_3 = c + [(3-d)/(b-d) \times (a-c)]$

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

a = lowest concentration giving stimulation index >3
b = actual stimulation index caused by a
c = highest concentration failing to produce a stimulation index of 3
d = actual stimulation index caused by c

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1.

The animal treated with the undiluted test material was humanely killed, post dose on Day 3, and the animal treated with the test material at a concentration of 50% v/v in acetone/olive oil 4:1 was humanely killed, on Day 4, due to the occurrence of clinical signs of toxicity that approached the moderate severity limit set forth in the UK Home Office Project Licence. Signs of systemic toxicity noted were hunched posture, lethargy, ptosis, splayed gait, fasciculations and increased respiratory rate.

No signs of systemic toxicity were noted in the animal treated with the test material at a concentration of 25% v/v in acetone/olive oil 4:1.

Based on this information the dose levels selected for the main test were 25%, 10% and 5% v/v in acetone/olive oil 4:1.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per lymph nodes for each individual animal and the stimulation index are given in Table 2.

A stimulation index of greater than 3 was recorded for the test material at a concentration of 25% v/v in acetone/olive oil 4:1.

A stimulation index of less than 3 was recorded for the test material at concentrations of 10% and 5% v/v in acetone/olive oil 4:1.

A stimulation index of greater than 3 was recorded for the positive control material at a concentration of 15% v/v in acetone/olive oil 4:1.

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 3.

There were no deaths. No signs of systemic toxicity were noted in the test animals, the vehicle control animals or the positive control animals during the test.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 4.

Bodyweight changes of the test animals or positive control animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CALCULATION OF EC_3 VALUE

$EC_3 = c + [(3-d)/(b-d) \times (a-c)]$

$a = 25$
$b = 3.14$
$c = 10$
$d = 1.96$

$EC_3 = 10 + [(3-1.96)/(3.14-1.96) \times (25-10)] = 23.2$

The concentration of test material expected to cause a 3 fold increase in ^{3}HTdR incorporation (EC_3 value) was calculated to be 23.2% v/v in acetone/olive oil 4:1.

7. CONCLUSION

The test material was considered to be a sensitiser under the conditions of the test.

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration (% v/v) in acetone/olive oil 4:1	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
100	S-1	21	-	0	0	0	0	0	HLPt Ws (18g) X*	-	-	-
50	S-2	21	-	0	0	0	0	0	0	HL Ws FaRi (20g) X*	-	-
25	S-3	20	21	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity
H = Hunched posture
L = Lethargy
Pt = Ptosis
Ws = Splayed gait
Fa = Fasciculations
Ri = Increased respiratory rate
( ) = Bodyweight prior to termination
X* = Animal humanely killed due to the occurrence of clinical signs of toxicity that approached the moderate severity limit set forth in the UK Home Office Project Licence
- = No data, animal dead

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 2 Individual Disintegrations per Minute and Stimulation Indices

Treatment Group	Animal Number	dpm/ Animal [a]	Mean dpm/Animal (Standard Deviation)	Stimulation Index [b]	Result
Vehicle acetone/olive oil 4:1	1-1	695.52	1210.12 (±385.70)	N/A	N/A
	1-2	1100.75			
	1-3	1251.06			
	1-4	1230.85			
	1-5	1772.41			
Test Material 5% v/v in acetone/olive oil 4:1	2-1	1944.76	1626.14 (±370.85)	1.34	Negative
	2-2	2108.83			
	2-3	1341.41			
	2-4	1391.61			
	2-5	1344.10			
Test Material 10% v/v in acetone/olive oil 4:1	3-1	1844.35	2371.38 (±1024.95)	1.96	Negative
	3-2	4136.21			
	3-3	2035.04			
	3-4	1539.66			
	3-5	2301.66			
Test Material 25% v/v in acetone/olive oil 4:1	4-1	3671.49	3796.20** (±683.02)	3.14	Positive
	4-2	3372.64			
	4-3	3656.07			
	4-4	3299.43			
	4-5	4981.39			
Positive Control Material 15% v/v in acetone/olive oil 4:1	5-1	3901.87	6106.55* (±2007.32)	5.05	Positive
	5-2	5463.77			
	5-3	5193.07			
	5-4	6775.23			
	5-5	9198.80			

dpm = Disintegrations per minute
a = Total number of lymph nodes per animal is 2
b = Stimulation Index of 3.0 or greater indicates a positive result
N/A = Not applicable
** = Significantly different from control group $p<0.01$
* = Significantly different from control group $p<0.05$

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Individual Clinical Observations and Mortality Data

Treatment Group	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle acetone/olive oil 4:1	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0
Test Material 5% v/v in acetone/olive oil 4:1	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0
Test Material 10% v/v in acetone/olive oil 4:1	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0
Test Material 25% v/v in acetone/olive oil 4:1	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0
Positive Control Material 15% v/v in acetone/olive oil 4:1	5-1	0	0	0	0	0	0	0	0	0
	5-2	0	0	0	0	0	0	0	0	0
	5-3	0	0	0	0	0	0	0	0	0
	5-4	0	0	0	0	0	0	0	0	0
	5-5	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 4 Individual Bodyweights and Bodyweight Changes

Treatment Group	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle acetone/olive oil 4:1	1-1	22	22	0
	1-2	21	20	-1
	1-3	19	21	2
	1-4	19	18	-1
	1-5	21	21	0
Test Material 5% v/v in acetone/olive oil 4:1	2-1	19	19	0
	2-2	18	19	1
	2-3	18	20	2
	2-4	19	20	1
	2-5	20	21	1
Test Material 10% v/v in acetone/olive oil 4:1	3-1	23	22	-1
	3-2	19	20	1
	3-3	19	20	1
	3-4	19	19	0
	3-5	19	18	-1
Test Material 25% v/v in acetone/olive oil 4:1	4-1	22	21	-1
	4-2	19	21	2
	4-3	19	20	1
	4-4	19	20	1
	4-5	20	20	0
Positive Control Material 15% v/v in acetone/olive oil 4:1	5-1	20	21	1
	5-2	18	19	1
	5-3	20	22	2
	5-4	20	19	-1
	5-5	20	21	1

Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC

Test Material:	α-Hexylcinnamaldehyde, Tech, 85%
Safepharm Laboratories Project number:	0039/0944
Study dates:	05 July 2007 to 11 July 2007

Methods. One group of five animals was treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, Tech, 85% as a solution in acetone/olive oil 4:1 at a concentration of 15% v/v. A further control group of five animals was treated with acetone/olive oil 4:1 alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in acetone/olive oil 4:1	Stimulation Index	Result
15	3.36	Positive

Conclusion. α-Hexylcinnamaldehyde, Tech, 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Material	Concentration	Vehicle	Stimulation Index [a]	Classification [b]
0039/0881•	29/09/06	05/10/06	2,4-Dinitrobenzenesulfonic acid, sodium salt	5%, 10%, 25% w/w	propylene glycol	10.68, 23.80, 40.25	Positive
0039/0911•	17/01/07	07/02/07	Phenylacetaldehyde (90%)	5%, 10%, 25% v/v	propylene glycol	11.25, 20.00, 29.49	Positive
0039/0931•	09/05/07	23/05/07	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	ethyl acetate	4.44, 7.97, 14.55	Positive
0039/0944•	05/07/07	11/07/07	α-Hexylcinnamaldehyde	15% v/v	acetone/olive oil 4:1	3.36	Positive
0039/0950•	23/08/07	29/08/07	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	dimethyl formamide	2.11, 2.82, 6.37	Positive
0039/0951•	24/08/07	30/08/07	α-Hexylcinnamaldehyde	10%, 25% v/v	butanone	2.86, 5.74	Positive
0039/0952•	05/09/07	11/09/07	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	dimethyl sulphoxide	1.84, 3.75, 5.59	Positive
0039/0953•	06/09/07	12/09/07	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	acetone	2.25, 3.56, 7.64	Positive
0039/0954•	10/08/07	16/08/07	α-Hexylcinnamaldehyde	5%, 10%, 25% v/v	ethanol/distilled water 7:3	1.38, 2.03, 8.04	Positive
0039/0955•	07/09/07	13/09/07	2,4-Dinitrobenzenesulfonic acid	1%, 5%, 10% w/w	1% pluronic L92 in distilled water	1.80, 4.32, 11.98	Positive
0039/0956•	20/09/07	26/09/07	Phenylacetaldehyde (90%)	1%, 2.5%, 5% v/v	propylene glycol	0.82, 2.67, 22.96	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result
• = Standard Test Method 599 ('Individual' nodes)

Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 ml test material + 0.5 ml vehicle	Vortex mixer	solution	suitable for dosing
dimethyl formamide	50% 0.5 ml test material + 0.5 ml vehicle	Vortex mixer	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

Appendix 4 Copy of Protocol and Protocol Amendment

SafePharm Laboratories

PROTOCOL

TEST MATERIAL	:	FR-1435P
STUDY TYPE	:	Local Lymph Node Assay in the Mouse
TEST METHOD	:	599.06
PROJECT NUMBER	:	0466/0301
PROPOSED START DATE	:	Mid December 2007
PROPOSED COMPLETION DATE	:	Early February 2008
TARGET (DRAFT) REPORT DATE	:	Early March 2008
SPONSOR	:	Bromine Compounds Ltd. Makleff House P.O.B. 180 BEER-SHEVA 84101 ISRAEL
SPECIAL CONDITIONS	:	An additional group of animals will be treated with a known moderate sensitiser. The identity of the positive control material will be documented in the study file.

This study is compliant with the following toxicology guidelines:

- OECD Guideline for the Testing of Chemicals 429, 2002
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Directive 2004/73/EC
- US EPA OPPTS 870.2600, 2003

APPROVED FOR SPONSOR BY: ..... DATE: 5/12/07

AUTHORISED BY: ..... DATE: 06 DEC 2007
A Sanders
STUDY DIRECTOR

This protocol is issued without signature by the Study Director to enable changes to be made if necessary prior to authorisation. Sponsors should sign and return the document to indicate approval and GLP authorisation will be confirmed by the Study Director's signature prior to the start of the study.

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION AND OBJECTIVES

The test is designed to assess the skin sensitising potential (delayed type hypersensitivity) of the test material in the mouse following topical application to the dorsal surface of the ear. Primary lymphocyte proliferation is assessed during the sensitising (induction) phase of the response. Lymphocyte proliferation in lymph nodes, from individual animals, draining the application site of the test material is quantified by measuring the incorporation of radiolabelled thymidine by β-Scintillation Counting.

The Local Lymph Node Assay (LLNA) can be used in the assessment of skin sensitisation potential as indicated in the OECD Guideline for the Testing of Chemicals, No 429, 2002 "Skin Sensitisation: Local Lymph Node Assay", Method B.42 of Commission Directive 2004/73/EC and to the EPA OPPTS Guideline, No 870.2600, "Skin Sensitisation", 2003.

The work will be performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106) as amended by SI 2004/0994). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM (98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

2. TEST FACILITY

Safepharm Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

3. ANIMALS

Specification

Female CBA/Ca strain mice obtained from an accredited breeder which will be detailed in the study data. At the start of the study the mice will weigh 15 to 23g and will be 8 to 12 weeks old.

Number

A minimum of five animals per dose group are used.

Justification

Preferred species of choice since quantitative methods have been developed for the measurement of skin sensitisation responses in the mouse.

4. ANIMAL HUSBANDRY

Environment

Target temperature:	19 to 25°C
Target humidity:	30 to 70%
Lighting:	Twelve hours of continuous artificial light in each twenty-four hour period
Ventilation:	at least fifteen air changes per hour

Housing

Individually housed, in suspended polypropylene cages fitted with stainless steel mesh lids and furnished with softwood woodflakes. Results of routine analysis of the woodflakes are made available to Safepharm Laboratories Ltd.

Diet and Water

Certified rat and mouse diet obtained from an accredited supplier and detailed in the report and mains tap water *ad libitum*.

The diet and water are routinely analysed and are considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study.

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

5. ANIMAL WELFARE

Environmental Enrichment

Animals will be provided with suitable environmental enrichment items.

Study Conduct

The study was designed and will be conducted to cause the minimum suffering or distress to the animals consistent with the scientific objectives and in accordance with the Safepharm policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedures) Act 1986. This standard test method is subject to review and the conduct of the study may be retrospectively reviewed, as part of the Safepharm Ethical Review Process.

6. INITIAL CONSIDERATIONS

Preliminary Screening Test

In the absence of toxicological information regarding irritation and/or systemic toxicity, or if the information suggests that irritation and/or toxicity is likely, then a preliminary screening test will be conducted with one animal.

The animal will be treated with the test material at the maximum soluble concentration in the most suitable vehicle (unless information is available to suggest that this concentration will be irritant or toxic. In such case, a lower concentration may be investigated). The animal will be treated, as detailed in the procedures section, for three consecutive days (Days 1, 2 and 3). The animal will be observed for five days from initiation of treatment. Any clinical signs of toxicity, or irritation at the treatment sites will be recorded. In the event of any clinical signs of toxicity or excessive skin irritation being noted, a further screening test will be performed at a lower concentration.

7. PRE-TEST PROCEDURES

Acclimatisation

At least five days.

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

Allocation

Animals will be allocated to studies using a total randomisation procedure and caged individually.

Identification

Each animal will be identified using indelible tail markings. A cage card will be prepared with details of test material, project number, dose level, sex, number of animal, study dates and initials of the Study Director.

8. TEST MATERIAL AND EXPERIMENTAL PREPARATION

Supply

Supplied by the Sponsor with details of hazardous properties if known. Data relating to the identification, purity and stability of the test material will be the responsibility of the Sponsor.

Storage

Room temperature in the dark unless otherwise specified by Sponsor.

Preparation

The test material will be prepared in a suitable vehicle at the required concentrations. The vehicle should be selected on the basis of maximising the concentration and solubility whilst producing a solution/suspension suitable for application of the test material. Suitable vehicles for use (in order of preference) are; acetone:olive oil (4:1 v/v), dimethyl formamide, methylethylketone (2-butanone), and dimethylsulphoxide. Other vehicles such as ethanol:water (7:3 v/v), ethanol:diethylphthalate (1:3 v/v) 1% v/v pluronic L92 in distilled water, absolute ethanol or acetone may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test material is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

Analysis

All formulations will be used within two hours of preparation and will be assumed to be stable for this period unless specified otherwise by the Sponsor. The concentration and homogeneity of the formulations will not be determined by analysis.

Absorption

The absorption of the test material will not be determined.

9. SELECTION OF CONCENTRATIONS OF TEST MATERIAL

At least three consecutive concentrations will be selected from the following concentration series: 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1%.

Lower concentrations or intermediate concentrations may be investigated if considered necessary.

The concentrations used will be based upon previous experience, (ie, preliminary screening test data) expected toxicity of the test material and/or the physical properties of the test material eg solubility, viscosity.

10. PROCEDURE

Test Material Administration

Groups of five mice will be treated at each of the concentrations of the test material. The mice will be treated by daily application of 25 µl of the appropriate concentration of the test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test material will be administered using an automatic micropipette. The test substance will be spread over the entire dorsal surface of the ear using the tip of the pipette.

A further group of five mice will receive the vehicle alone in the same manner.

^{3}H-methyl thymidine Administration

Five days following the first topical application of the test material (Day 6) all mice will be injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml) giving a total of 20 µCi to each mouse.

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

11. OBSERVATIONS

Clinical Observations

All animals will be observed on a daily basis, including pre and post-dose observations on Days 1, 2 and 3. Any clinical signs of toxicity, skin irritation or signs of ill health during the study will be recorded.

Bodyweights

Recorded on Day 1 (prior to dosing) and prior to termination.

12. TERMINAL PROCEDURE

Termination

Five hours following the administration of ^{3}HTdR the mice will be killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes will be excised and processed (2 nodes per animal). In some circumstances it may not be possible to use all the mice from a particular group, eg. due to unsuccessful ^{3}H-methyl thymidine injection. In this situation the animal will be removed from the group and the assay performed with the remaining animals. This will be recorded in the study file. For each individual animal 1 ml of PBS will be added to the lymph nodes.

Preparation of Single Cell Suspensions

A single cell suspension of the lymph node cells for each individual animal will be prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells (LNC) will be rinsed through the gauze with 4 ml of PBS into a petri dish. The LNC suspension will be transferred to an appropriately sized centrifuge tube. The petri dish will be washed with an additional 5 ml of PBS to remove all remaining cells, this will be added to the centrifuge tube. The lymph node cells will be pelleted at 1400 rpm (approximately 190g) for 10 minutes. The pellet will be re-suspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet will be re-suspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation

After an incubation period of approximately 18 hours at approximately 4°C, the precipitates will be recovered by centrifugation at 2100 rpm (approximately 450g) for 10 minutes, followed by resuspension in 1 ml of TCA. The cell suspension of each individual animal will be transferred to a "Poly Q™" vial

containing 10 ml of scintillation fluid. The vials will be placed in the sample changer of the scintillator and allowed to stand in darkness for approximately 20 minutes. This period of time in the dark will reduce the risk of luminescence, which can affect the reliability of the results. On completion of this period the vials will be shaken vigorously, preferably in darkness. ^{3}HTdR incorporation will be measured by β-scintillation counting. For each individual animal the proliferative response of LNC's will be expressed as the number of radioactive disintegrations per minute per animal (dpm/animal). The mean dpm/animal will be determined for each test group and the vehicle control group. The stimulation index (SI) for each test concentration is calculated by dividing the mean dpm/animal of each test group by the dpm/animal of the vehicle control group.

13. STATISTICAL ANALYSIS

Appropriate statistical analysis of the data will be performed using commercially available software.

14. INTERPRETATION OF RESULTS

The decision process to identify a positive response will include consideration of the SI (especially SI's ≥3), dose response and statistical significance.

15. POSITIVE CONTROL DATA

Studies conducted to EPA OPPTS Guidelines 870.2600 will require a concurrent positive control group to be included in each test. Positive controls are used to check the sensitivity and reliability of the test system. The positive control must produce a positive LLNA response at a concentration level expected to give an increase in the stimulation index (SI) of three or greater over the vehicle control group. The positive control dose is to be such that the induction is clear but not excessive. Preferred positive control substances are α-hexyl cinnamicaldehyde, (CAS No. 101-86-0) and mercaptobenzothiazole (CAS No. 149-30-4). There may be circumstances where, given adequate justifications, other positive control substances may be used.

The positive control substance is tested in a vehicle that is known to produce a consistent response (eg 4:1 acetone/olive oil).

The sensitivity and reliability of the test system will be checked at an interval no greater than 6 months.

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

16. QUALITY ASSURANCE

This standard test method will be reviewed for GLP compliance and the final report will be audited by Safepharm Quality Assurance Unit. This type of study is subject to process-based QA inspection designed to encompass the major phases once per month.

17. PROTOCOL AMENDMENTS

Amendments to protocol will be made only by completion of an Amendment to Protocol form authorised by the Study Director.

18. FINAL REPORT

The final report will include, as a minimum, the following information:

Species, strain of animals
Experimental design
The dpm/animal for each animal in the study. The mean dpm/animal for each group and the test/control ratio (stimulation index) for each dosage group in tabular form
Classification of sensitisation potential

19. ARCHIVE

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years after which instructions will be sought as to further retention or disposal. Further retention or return will be chargeable to the Sponsor.

FR-1435P : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 4 (continued) Copy of Protocol and Protocol Amendment

SAFEPHARM LABORATORIES LIMITED

AMENDMENT TO PROTOCOL

AMENDMENT NUMBER:	One
PROTOCOL TITLE:	Local Lymph Node Assay in the Mouse
TEST MATERIAL:	FR-1435P
PROJECT NUMBER:	0466/0301
TEST METHOD:	599.06
SPONSOR:	Bromine Compounds Ltd. Makleff House P.O.B. 180 BEER-SHEVA 84101 ISRAEL
AMENDMENT:	α-Hexylcinnamaldehyde, at a concentration of 15% v/v in acetone/olive oil 4:1, will be used for treatment of the positive control group.

AUTHORISED FOR SAFEPHARM LABORATORIES LIMITED BY: ..... DATE: 30 JAN 2008

A Sanders
STUDY DIRECTOR

Appendix 5 Certificate of Analysis

ICL Industrial Products

IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel:972-4-8469563 Fax:972-4-8469320 zilbermanj@tami-imi.icl-ip.com

October 18, 2007

Certificate of Analysis

CONFIDENTIAL

Batch 38250-29-6	
Appearance, Color	Yellow-amber liquid
Assay, % (GC area)	>98
Water, ppm	<500
Br total, %	63
Density, g/cm^3 at 25°C	~2.0
Viscosity, cps at 23°C	1800-2400
Acidity, meq/g	<0.1
Solubility, g/100 g Water Toluene	 <0.1 complete
Expiration date	18.10.08

Appendix 6 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **Shardlow** **Derbyshire** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem testing** **Toxicology**

DATE OF INSPECTION

21st August 2007

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

15/10/07

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

- Attachment 12 -

REPORT

Study Title

ASSESSMENT OF ACUTE DERMAL TOXICITY WITH FR-1435X IN THE RAT

Author

Drs. A.H.B.M. van Huygevoort

Study completion date

02 June 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419862
NOTOX Substance 146232/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Drs. A.H.B.M. van Huygevoort
Study Director

Date: 2 June 2005

Drs. M.S. Teunissen
Section Head Toxicology

Date: 09 June 2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected

Type of inspections	Phase/Process	Start inspection date	End Inspection date	Reporting date
Study	Protocol	14-OCT-04	14-OCT-04	14-OCT-04
	Amendment 2 of protocol	18-JAN-05	18-JAN-05	18-JAN-05
	Amendment 3 of protocol	23-FEB-05	23-FEB-05	23-FEB-05
	Amendment 4 of protocol	02-MAR-05	02-MAR-05	02-MAR-05
	Amendment 5 of protocol	24-MAY-05	24-MAY-05	24-MAY-05
	Report	07-MAR-05	07-MAR-05	07-MAR-05
Process	**Test substance unit**			
	Test substance weighing	01-NOV-04	11-NOV-04	11-NOV-04
	Test substance formulation	01-NOV-04	11-NOV-04	11-NOV-04
	SPF unit			
	Rat/mouse exposure	01-NOV-04	11-NOV-04	11-NOV-04
	Rat/Mouse Observation/Measurement	01-NOV-04	11-NOV-04	11-NOV-04
	Pathology unit			
	Rat/mouse necropsy	03-NOV-04	17-NOV-04	17-NOV-04

Head of Quality Assurance
C.J. Mitchell B.Sc.

I.A.

Date: 13-JAN-2005

4. SUMMARY

Assessment of acute dermal toxicity with FR-1435X in the rat.

The study was carried out based on the guidelines described in: "Acute Toxicity-Dermal", OECD No.402 (1987); "Acute Dermal Toxicity", EC Commission Directive 92/69/EEC, Part B.3 (1992); Environmental Protection Agency (EPA): Health Effects Test Guidelines OPPTS 870.1200 (1998), "Acute Dermal Toxicity" and JMAFF: Japanese Test Guidelines (2000).

FR-1435X was administered to five Wistar rats of each sex by a single dermal application at 2000 mg/kg body weight for 24 hours. Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed after terminal sacrifice (day 15).

No mortality occurred.

Chromodacryorrhoea on the snout and/or ptosis were noted in some animals on day 1.
Scales were seen in the treated skin-area of three females between days 3 and 10.

The body weight gain during the observation period was within the range expected for rats used in this type of study.

Macroscopic post mortem examination of the animals did not reveal any test substance related abnormalities.

The dermal LD_{50} value of FR-1435X in Wistar rats was established to exceed 2000 mg/kg body weight.

Based on the lack of adverse reactions following a single dermal application to the Wistar rats at a limit test dose level of 2000 mg/kg body weight for 24 hour exposure duration, the test item FR-1435X does not represent an acute toxicity by the dermal route.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start : 20 October 2004 Completion : 03 November 2004

5.2. Aims of the study

The objective of this study was to assess the toxicity of the test substance when administered to rats as a single dermal application.
This study should provide a rational basis for risk assessment in man.
The dermal route was selected as it is a possible route of human exposure during manufacture, handling or use of the test substance.

5.3. Guidelines

As required by the Dutch Act on Animal Experimentation (February 1997), the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-13). The study procedures described in this report were based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No. 402, "Acute Dermal Toxicity", Paris Cedex, 1987.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the determination of Toxicity, as last amended by Commission Directive 92/69/EEC, B.3: "Acute Toxicity-Dermal". Official Journal of the European Communities No. L 383, 1992.

United States Environmental Protection Agency (EPA). Health Effects Test Guidelines, OPPTS 870.1200, Acute Dermal Toxicity. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-96-192, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000, including the most recent partial revisions.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test Substance

The sponsor is responsible for all test substance data unless determined by NOTOX.

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	
Polyethylene glycol	Not indicated

6.1.2. Test substance preparation

Vehicle	Polyethylene glycol 400 (specific gravity 1.125)
Rationale	The vehicle was selected based on trial formulations performed at NOTOX.
Preparation	The formulation (w/w) was prepared within 4 hours prior to dosing. Homogeneity was accomplished to a visually acceptable level. Adjustment was made for specific gravity of the vehicle.

6.2. Test System

Species	Rat, Wistar strain Crl:(WI) BR (outbred, SPF-Quality). Recognised by international guidelines as the recommended test system (e.g. OECD, EC). Source: Charles River Deutschland, Sulzfeld, Germany.
Number of animals	5 males and 5 females (females were nulliparous and non-pregnant).
Age and body weight	Young adult animals (approx. 9 weeks old) were selected. Body weight variation did not exceed +/- 20% of the sex mean.
Identification	Earmark

6.3. Animal husbandry

Conditions
Animals were housed in a controlled environment, in which optimal conditions were considered to be approximately 15 air changes per hour, a temperature of 21.0 ± 3.0°C (actual range: 19.1 - 21.9°C), a relative humidity of 30-70% (actual range: 38 - 75%) and 12 hours artificial fluorescent light and 12 hours darkness per day.
Cleaning procedures in the room might have caused the temporary fluctuations above the optimal maximum level of 70% for relative humidity. Based on laboratory historical data, these fluctuations were considered not to have affected the study integrity.

Accommodation
Individually housed in labelled Macrolon cages (type III, height 15 cm.) containing sterilised sawdust as bedding material (Woody-Clean type 3/4; Tecnilab-BMI BV, Someren , The Netherlands) and paper as cage-enrichment (Enviro-dri, BMI, Helmond, The Netherlands). Certificates of analysis were examined and then retained in the NOTOX archives.
Acclimatisation period was at least 5 days before start of treatment under laboratory conditions.

Diet
Free access to standard pelleted laboratory animal diet (from Altromin (code VRF 1), Lage, Germany). Certificates of analysis were examined and then retained in the NOTOX archives.

Water
Free access to tap-water. Certificates of quarterly analysis were examined and then retained in the NOTOX archives.

Results of analysis for ingredients and/or contaminants of diet, sawdust, paper, and water were assessed and did not reveal any findings that were considered to have affected study integrity.

6.4. Treatment

A health inspection was performed prior to commencement of treatment, to ensure that the animals were in a good state of health. Special attention was paid to the skin to be treated, which was intact and free from any abnormality.

Method	Dermal application.
Clipping	One day before exposure (day -1) an area of approximately 5x7 cm on the back of the animal was clipped.
Application	The formulation was applied in an area of approx. 10% of the total body surface, i.e. approx. 25 cm² for males and 18 cm² for females. The formulation was held in contact with the skin with a dressing, consisting of a surgical gauze patch (Surgy 1D)*, successively covered with aluminium foil and Coban elastic bandage*. A piece of Micropore tape* was additionally used for fixation of the bandages in females only. *. Manufacturers: Laboratoires Stella s.a., Liege, Belgium (surgical gauze) and 3M, St. Paul, Minnesota, U.S.A. (Coban & Micropore).
Frequency	Single dosage, on day 1.
Dose level (volume)	2000 mg/kg (10 ml/kg) body weight.
Application period	24 hours, after which dressings were removed and the skin cleaned of residual test substance using water.

6.5. Observations

Mortality/Viability	Twice daily.
Body weights	Days 1 (pre-administration), 8 and 15.
Clinical signs	At periodic intervals on the day of dosing (day 1) and once daily thereafter, until day 15. The time of onset, degree and duration were recorded and the symptoms graded according to fixed scales: Maximum grade 4: grading slight (1) to very severe (4) Maximum grade 3: grading slight (1) to severe (3) Maximum grade 1: presence is scored (1).
Necropsy	At the end of the observation period, all animals were sacrificed by asphyxiation using an oxygen/carbon dioxide procedure and subjected to necropsy. Descriptions of all internal macroscopic abnormalities were recorded.

6.6. Interpretation

No formal interpretation was done.

6.7. List of protocol deviations

There were no deviations from the protocol.

7. RESULTS

7.1. Mortality

No mortality occurred.

7.2. Clinical Signs (Table 1)

Chromodacryorrhoea on the snout and/or ptosis were noted in some animals on day 1. Scales were seen in the treated skin-area of three females between days 3 and 10.

7.3. Body Weight (Table 2)

The changes noted in body weight gain in males and females were within the range expected for rats used in this type of study and were therefore considered not indicative of toxicity.

7.4. Macroscopic Findings (Table 3)

Macroscopic post mortem examination of the animals did not reveal any test substance related abnormalities.

Incidental findings included diaphragmatic hernia of the liver and a reduced size of the left kidney in one female. These findings are commonly noted among rats of this age and strain and therefore considered not toxicologically significant.

8. CONCLUSION

The dermal LD_{50} value of FR-1435X in Wistar rats was established to exceed 2000 mg/kg body weight.

Based on the lack of adverse reactions following a single dermal application to the Wistar rats at a limit test dose level of 2000 mg/kg body weight for 24 hour exposure duration, the test item FR-1435X does not represent an acute toxicity by the dermal route.

TABLE 1 : CLINICAL SIGNS

Individual clinical signs observed through the entire 15 day observation period in male and female Wistar rats, following a single dermal application of FR-1435X, at dose level of 2000 mg/kg body weight.

TEST DAY HOURS AFTER TREATMENT	MAX GRADE	1 0	1 2	1 4	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MALES 2000 MG/KG																		
ANIMAL 1																		
Secretion / excretion																		
Chromodacryorrhoea (Snout)	(3)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Various																		
Ptosis	(3)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 2																		
No clinical signs noted		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 3																		
No clinical signs noted		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 4																		
Secretion / excretion																		
Chromodacryorrhoea (Snout)	(3)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 5																		
No clinical signs noted		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FEMALES 2000 MG/KG																		
ANIMAL 6																		
Skin / fur / plumage																		
Scales (Treated skin)	(3)	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 7																		
Skin / fur / plumage																		
Scales (Treated skin)	(3)	-	-	-	-	-	-	-	1	1	1	1	1	-	-	-	-	-
ANIMAL 8																		
Skin / fur / plumage																		
Scales (Treated skin)	(3)	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 9																		
Secretion / excretion																		
Chromodacryorrhoea (Snout)	(3)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ANIMAL 10																		
No clinical signs noted		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = SIGN NOT OBSERVED / . = OBSERVATION NOT PERFORMED / + = ANIMAL DEAD

TABLE 2 : BODY WEIGHTS (GRAM).
Individual and mean (± SD) group values of body weights and gain (gram) in male and female Wistar rats, following a single dermal application of FR-1435X, at dose level of 2000 mg/kg body weight.

SEX/DOSE LEVEL	ANIMAL	BODYWEIGHTS DAY 1	DAY 8	DAY 15	BODY WEIGHT GAIN WEEK 1	WEEK 2
MALES 2000 MG/KG						
	1	313	340	390	27	50
	2	282	313	357	31	44
	3	291	319	353	28	34
	4	309	350	396	41	46
	5	333	370	421	37	51
	MEAN	306	338	383	33	45
	ST.DEV.	20	23	28	6	7
	N	5	5	5	5	5
FEMALES 2000 MG/KG						
	6	202	217	221	15	4
	7	215	224	250	9	26
	8	225	245	261	20	16
	9	217	245	264	28	19
	10	212	222	249	10	27
	MEAN	214	231	249	16	18
	ST.DEV.	8	13	17	8	9
	N	5	5	5	5	5

TABLE 3 : MACROSCOPIC FINDINGS.
Macroscopic findings in in male and female Wistar rats, 15 days following a single dermal application of FR-1335X, at dose level of 2000 mg/kg body weight.

ANIMAL	ORGAN	FINDING	DAY OF DEATH
MALES 2000 MG/KG			
1		No findings noted	Scheduled necropsy Day 15 after treatment
2		No findings noted	Scheduled necropsy Day 15 after treatment
3		No findings noted	Scheduled necropsy Day 15 after treatment
4		No findings noted	Scheduled necropsy Day 15 after treatment
5		No findings noted	Scheduled necropsy Day 15 after treatment
FEMALES 2000 MG/KG			
6	Liver	Diaphragmatic hernia.	Scheduled necropsy Day 15 after treatment
	Kidneys	Left side: reduced in size.	
7		No findings noted	Scheduled necropsy Day 15 after treatment
8		No findings noted	Scheduled necropsy Day 15 after treatment
9		No findings noted	Scheduled necropsy Day 15 after treatment
10		No findings noted	Scheduled necropsy Day 15 after treatment

Appendix 1

GLP Certificate

[redacted]

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 8-12, 17 and 23 April, 1 and 3 May 2002 at

Notox
Safety & Environmental Research B.V.
Hambakenwetering 7, P.O. Box 3476
5203 DL 's Hertogenbosch

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Physical-chemical testing; Toxicity studies; Mutagenicity studies; Environmental toxicity studies on aquatic and terrestrial organisms; Studies on behaviour in water, soil, and air, bioaccumulation; Residue studies; Analytical and clinical chemistry testing; Toxicokinetic studies; In-vitro metabolism tests.

The Hague, 16 September 2002

NEDERLAND

[redacted] Helder, DVM
GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Ministry of Health, Welfare and Sport

Appendix 2

Certificate of Analysis

ICL Industrial PRODUCTS

IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel:972-4-8469553 Fax:972-4-8469320 zilbermanj@tami-imi.icl-ip.com

Certificate of Analysis

FR-1435X

Brominated alkyl aryl compound

Batch No.	38278-53-4
Appearance, Color	Brown liquid
Assay, % (HPLC area)	Min. 97
Water, ppm	<500
Br total, %	63
Density, kg/m^3 at 25°C	~2000
Viscosity, cps at 23°C	1600-2000
Acidity, meq/g	<0.1
Solubility, g/100 g Water Toluene	 <0.1 complete

Appendix 3

Copy of protocol and amendments

ORIGINAL

PROTOCOL

Study Title

ASSESSMENT OF ACUTE DERMAL TOXICITY WITH FR-1435X IN THE RAT

Author

Drs. A.H.B.M. van Huygevoort

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419862
NOTOX Substance 146232/A

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FR-1435X NOTOX Project 419862

1. CONTENTS

FR-1435X NOTOX Project 419862

2. PROTOCOL APPROVAL

STUDY DIRECTOR: Drs. A.H.B.M. van Huygevoort

date: 8 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.

date: 12/10/04.

SPONSOR: Dead Sea bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

date: 18.10.04

FR-1435X NOTOX Project 419862

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan (in-life phase)	Start week beginning :18 October 2004 (week 43) Completed week beginning :29 November 2004 (week 49) Proposed draft reporting date: 13 February 2005 (week 06)

3.2. Aims of study

The objective of this study is to assess the toxicity of the test substance when administered to rats as a single dermal dose. The results of the study allow the test substance to be ranked according to most classification systems, currently in use.
This study should provide a rational basis for risk assessment in man. The dermal route is selected, as it is a possible route of human exposure during manufacture, handling or use of the test substance.

3.3. Guidelines

This protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 97-03-13) as required by the Dutch Act on Animal Experimentation (February 1997). The study procedures described in this protocol are based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No. 402, "Acute Dermal Toxicity", Paris Cedex, 1987.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the determination of Toxicity, as last amended by Commission Directive 92/69/EEC, B.3: "Acute Toxicity-Dermal". Official Journal of the European Communities No. L 383, 1992.

United States Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.1200, Acute Dermal Toxicity. Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-96-192, August 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF), 12 Nousan, Notification No 8147, November 2000; including the most recent partial revisions.

FR-1435X NOTOX Project 419862

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

FR-1435X NOTOX Project 419862

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

The sponsor is responsible for all test substance data unless determined by NOTOX.
All available information on characteristics of the test substance will be taken into account before start of the study in order to determine if they conflict with/might affect the proceeding of the study.

CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Oensity	~2000
Stability in vehicle	
Water	Not indicated
1% Aq. Carboxymethyl cellulose	Not indicated
Corn oil	Not indicated
Propylene glycol	Not indicated
Polyethylene glycol	Not indicated
Methyl ethyl ketone	Not indicated
Olmethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Olive oil	Not indicated
Olmethyl formamide	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

FR-1435X NOTOX Project 419862

4.1.2. Test substance preparation

If practically possible, the test substance will be dosed as delivered by the sponsor. If not, a suitable vehicle will be selected, based on test substance data provided by the sponsor and/or trial formulation results. The vehicle will be chosen from (in order of preference): water (Milli-U), 1% aq. carboxymethyl cellulose, propylene glycol (spec.gravity 1.036), polyethylene glycol 400 (spec. gravity 1.125) and corn oil (spec. gravity 0.92) and approved by the Study Director in the study files.
Formulations (w/w) will be prepared within 4 hours prior to dosing and will be homogenised to visually acceptable levels. If applicable, adjustment will be made for specific gravity of vehicle. In case of multiple dose levels, the concentration of the test substance in vehicle will be varied to allow constant dosage volume in terms of ml/kg body weight.

4.2. Test system

Species	Rat: Wistar Crl: (WI) BR (outbred, SPF-Quality). Recognised by international guidelines as recommended test system (e.g. OECD, EC). Source: Charles River Deutschland, Sulzfeld, Germany.
Number of animals	5 males and 5 females per dose group (females will be nulliparous and non-pregnant).
Age and body weight	Young adult animals will be selected (8-12 weeks old). Animals to be used within the study will be of approx. the same age and body weight variation will not exceed +/- 20% of the sex mean.
Identification	Earmark

4.3. Animal husbandry

Conditions
A controlled environment is maintained in the room with optimal conditions of approximately 15 air changes per hour, a temperature of 21.0±3.0°C, a relative humidity of 30-70% and a 12 hour light/12 hour dark cycle. Temporary deviations from the maximum level for relative humidity (with a maximum of 20%) and light/dark cycle (with a maximum of 1 hour) may occur due to cleaning procedures or performance of functional observations in the room. Based on laboratory historical data these deviations are considered not to affect the study integrity.

Accommodation
Individually housed in labeled Macrolon cages (type III; height 15 cm.) containing purified sawdust as bedding material (Woody-Clean type 3/4; Tecnilab-BMI BV, Someren , The Netherlands) and paper as cage-enrichment (Enviro-dri, BMI, Helmond, The Netherlands). Certificates of analysis are examined and then retained in the NOTOX archives. The acclimatisation period will be at least 5 days before the start of treatment under laboratory conditions.

Diet
Free access to standard pelleted laboratory animal diet (from Altromin (code VRF 1), Lage, Germany). Certificates of analysis are examined and then retained in the NOTOX archives.

Water
Free access to tap-water. Certificates of quarterly analysis are examined and then retained in the NOTOX archives.

FR-1435X NOTOX Project 419862

4.4. Treatment

A health inspection will be performed prior to commencement of treatment, to ensure that the animals are in a good state of health. Special attention will be paid to the skin to be treated, which will be intact and free from any abnormality.

Method	Dermal application.
Clipping	One day before treatment (day -1), an area of approximately 5x7 cm on the back of the animals will be clipped.
Application	The test substance will be applied in an area of approx. 10% of the total body surface, i.e. approx. 25 cm^2 for males and 18 cm^2 for females. The test substance will be held in contact with the skin with a dressing, consisting of a surgical gauze patch (Surgy 1D)*, successively covered with aluminium foil and Coban elastic bandage*. A piece of Micropore tape* will additionally be used for fixation of the bandages in females only. *. Manufacturers: Laboratoires Stella s.a., Liege, Belgium (surgical gauze) and 3M, St Paul, Minnesota, U.S.A. (Coban & Micropore).
Frequency	Single dosage, on day 1.
Highest dose level	2000 mg/kg body weight. However, other dose levels (specified and approved by the Study Director in the study files) may be used to satisfy the requirements of the study.
Dose volume If dosed as such	Dose volume (ml/kg body weight) will be calculated as follows: Dose level (g/kg) / spec.gravity or density (g/ml). The density will be determined at trial formulation, if unknown, and approved by the Study Director in the study files.
If a vehicle is used	10 ml formulation/kg body weight.
Application period	24 hours, after which the dressing will be removed and the skin cleaned of residual test substance using water or an appropriate vehicle.

FR-1435X NOTOX Project 419862

4.5. Observations

Mortality/Viability	At least twice daily. Animals showing pain, distress or discomfort, which is considered not transient in nature or is likely to become more severe, will be sacrificed for humane reasons based on OECD guidance document on humane endpoints (ENV/JM/MONO/ 2000/7). The time of death will be recorded as precisely as possible.
Body weights	On days 1 (pre-administration), 8 and 15 and at death (if found dead after day 1).
Clinical signs	At periodic intervals on the day of dosing (at least three times) and once daily thereafter. The observation period will be 15 days. If clinical signs are present or expected to be present on day 15, the Study Director will consider the necessity of an extension of the observation period. All symptoms will be graded according to fixed scales (specified in the report) and the time of onset, degree and duration will be recorded.
Necropsy	All moribund animals and animals surviving to the end of the observation period will be sacrificed by asphyxiation using an oxygen/carbon dioxide procedure. All animals assigned to the study will be subjected to necropsy and descriptions of all internal macroscopic abnormalities will be recorded.

4.6. Interpretation

The results will be evaluated according to the OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998) and the EC criteria for classification and labelling of dangerous substances and preparations (Council Directive 67/548/EEC and all adaptations to technical progress and amendments of this Directive published in the Official Journal of the European Communities).

5. DISTRIBUTION

Original:	Study Director
1 Copy:	Coordinating Biotechnician (via Study Director)
1 Copy:	QAU/Management
1 Copy:	Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title	Assessment of acute dermal toxicity with FR-1435x in the rat
Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A (file 146223)
NOTOX Project	419862

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1 Test substance information:

Description	Brown liquid

2. Page 4, paragraph 3.1 Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1.+ 2. The data has been updated according to information of the sponsor, dated January 06, 2005

APPROVAL
Study director

.....
Drs. A.H.B.M. van Huygevoort

14 January 2005
date:

Page 1 of 1

ORIGINAL

PROTOCOL AMENDMENT NO : 3

Study Title	Assessment of acute dermal toxicity with Fr-1435x in the rat
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419862

AMENDMENT DESCRIPTION

1. Page 6, paragraph 5.1 Test substance information:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

date: 25 February 2005

ORIGINAL

PROTOCOL AMENDMENT NO : 4

Study Title	Assessment of acute dermal toxicity with Fr-1435X in the rat
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshilz
NOTOX Substance	146232/A
NOTOX Project	419862

AMENDMENT DESCRIPTION

1. Protocol amendment No 1 does not exist.

REASONS FOR AMENDMENT

1. This amendment serves to clarify the numbering of the amendments.

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

date: 28 February 2005

ORIGINAL

PROTOCOL AMENDMENT NO : 5

Study Title	Assessment of acute dermal toxicity with Fr-1435X in the rat
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419862

AMENDMENT DESCRIPTION

1. No formal interpretation will be done

REASONS FOR AMENDMENT

1. The sponsor requested this

APPROVAL
Study director

Drs. A.H.B.M. van Huygevoort

25 May 2005
date:

- Attachment 13 -

REPORT

Study Title

EVALUATION OF THE MUTAGENIC ACTIVITY OF FR-1435X IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY (WITH INDEPENDENT REPEAT)

Author

C.M. Verspeek-Rip

Study completion date

11 January 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Sponsor

Bromine Compound Ltd.
P.O. Box 180
BEER-SHEVA 84101
Israel

Laboratory Project Identification

NOTOX Project 419906
NOTOX Substance 146232/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

C.M. Verspeek-Rip
Study Director

Date: 11 January 2005

Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology

Date: 13/01/2005

3. QUALITY ASSURANCE STATEMENT

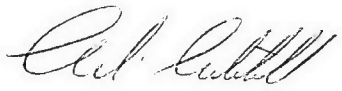
NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase / Section	Start Inspection date(s)	End Inspection date(s)	Reporting date
Protocol (Study)		05-10-2004	05-10-2004	05-10-2004
On-site (Process)	Genetic toxicology & *in vitro* metabolism	25-10-2004	28-10-2004	28-10-2004
Report (Study)		02-12-2004	02-12-2004	02-12-2004

Head of Quality Assurance
C.J. Mitchell B.Sc.



Date: 16-Dec-05

4. SUMMARY

Evaluation of the mutagenic activity of FR-1435X in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat).

FR-1435X was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA100 and TA98) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* WP_2uvrA. The test was performed in two independent experiments in the presence and absence of S9-mix (Aroclor-1254 induced rat liver S9-mix).

The study procedures described in this report were based on the most recent OECD, EEC, OPPTS and JMAFF guidelines.

Batch 38278-53-4 of FR-1435X was a brown liquid with a purity of 97.2%. The test substance was dissolved in dimethyl sulfoxide.

In the dose range finding test, FR-1435X was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix in the strains TA100 and WP_2uvrA. FR-1435X precipitated on the plates at dose levels of 3330 and 5000 µg/plate. The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

Based on the results of the dose range finding test, FR-1435X was tested in the first mutation assay at a concentration range of 33 to 3330 µg/plate in the absence and presence of 5% (v/v) S9-mix in tester strains TA1535, TA1537 and TA98. In the second mutation assay, FR-1435X was tested at the same concentration range in the absence and presence of 10% (v/v) S9-mix in tester strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. FR-1435X precipitated on the plates at the top dose of 3330 µg/plate. The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

FR-1435X did not induce a dose-related, two-fold increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in an independently repeated experiment.

In this study, the negative and strain-specific positive control values were within our laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that FR-1435X is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	C.M. Verspeek-Rip
Technical Coordinator:	Ing. A. Tijdink
Study Plan	Start : 22 October 2004 Completion : 08 November 2004

5.2. Aims of the study

The objective of this study was to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

Background of the test system
The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay have shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The *Salmonella typhimurium* strains used in this study were TA98, TA100, TA1535 and TA1537. The *Escherichia coli* strain used was WP_2uvrA.

The strains TA98 and TA1537 are capable of detecting frameshift mutagens, strains TA100, TA1535 and WP_2uvrA are capable of detecting base-pair substitution mutagens (Ref. 1, 2, 3, 4 and 5).

5.3. Guidelines

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test" (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria". EEC Publication Commission Directive (Published June 8, 2000).
- EPA Health Effects Test Guidelines, OPPTS 870.5100: Bacterial reverse Mutation Test. (August 1998)
- Guidelines stipulated by the Japanese Ministry of Agriculture, Forestry and Fisheries.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test Substance

CONFIDENTIAL

Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	Dimethyl sulfoxide: Not indicated

The sponsor is responsible for all test substance data unless determined by NOTOX. A certificate of analysis supplied by the sponsor is given in Appendix III.

6.1.2. Test Substance preparation

The test substance was dissolved in dimethyl sulfoxide of spectroscopic quality (Uvasol, Merck, Darmstadt, Germany). Test substance concentrations were used within 4 hours after preparation.

6.2. Reference substances

6.2.1. Negative control

The vehicle of the test article, being dimethyl sulfoxide.

6.2.2. Positive controls

Without metabolic activation (-S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1535	sodium azide (SA) (Sigma, Zwijndrecht, The Netherlands)	5 µg	Saline
TA1537	9-aminoacridine (9AC) (Janssen Chimica, Beers, Belgium)	60 µg	Milli-Q water
TA98	2-nitrofluorene (NF) (Merck)	10 µg	DMSO

TA100	methylmethanesulfonate (MMS) (Merck)	650 µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10 µg	DMSO

With metabolic activation (+S9-mix):
The positive control substance used for all tester strains was 2-aminoanthracene (2AA) (Sigma).
The following doses were used:

Strain	Concentration/plate	Amount of S9-mix	Solvent
TA1535	1 µg	5 and 10%	DMSO
TA1537	2.5 µg	5%	DMSO
TA1537	5 µg	10%	DMSO
TA98	1 µg	5 and 10%	DMSO
TA100	1 µg	5%	DMSO
TA100	2.5 µg	10%	DMSO
WP_2uvrA	10 µg	5 and 10%	DMSO

Solvents for reference substances
Saline = physiological saline (B. Braun, Melsungen AG, Germany)
DMSO = dimethyl sulfoxide of spectroscopic quality (Merck)
Milli-Q water (Millipore Corp., Bedford, MA., USA)

6.3. Test System

Test System	*Salmonella typhimurium* bacteria and *Escherichia coli* bacteria
Rationale	Recommended test system in international guidelines (e.g. OECD and EEC).
Source	*Salmonella typhimurium* strains: Dr. Bruce N. Ames, University of California at Berkeley, U.S.A. TA98 received on 21-02-1991, used batch: TA98.050204 TA1535 received on 30-07-2001, used batch: TA1535.050204 TA1537 received on 30-07-2001, used batch: TA1537.050204 Xenometric, Boulder, Co, U.S.A. (obtained from N.V. Organon) TA100 received on 19-09-2002, used batch: TA100.240604 *Escherichia coli* strain: Prof. Dr. B.A. Bridges, University of Sussex, Brighton, U.K. WP_2uvrA received on 23-10-1987, used batch: EC.160404

The characteristics of the different *Salmonella typhimurium* strains were as follows:

Strain	Histidine mutation	Mutation type
TA1537	*his*C3076	Frameshift
TA98	*his*D3052/R-factor*	Frameshift
TA1535	*his*G46	Base-pair substitutions
TA100	*his*G46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:
rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB : loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains were regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The sensitivity of the strain to a wide variety of mutagens has been enhanced by permeabilization of the strain using Tris-EDTA treatment (ref.1). The strain was regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196°C).

6.4. Cell culture

Preparation of bacterial cultures
Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid LTD, Hampshire, England) and incubated in a shaking incubator (37°C, 150 spm), until the cultures reached an optical density of 1.0 ± 0.1 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain were used for a test.

Agar plates
Agar plates (ø 9 cm) contained 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid LTD) in Vogel-Bonner Medium E, 20 g glucose (Merck).
N.B. The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin (Merck) and 15 µg/plate histidine (Merck) and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan (Sigma).

Top agar
Milli-Q water containing 0.6% (w/v) bacteriological agar (Oxoid LTD) and 0.5% (w/v) Sodium Chloride (Merck) was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at 121 ± 3°C.

Environmental conditions
All incubations were carried out in the dark at 37 ± 1 °C. The temperature was monitored during the experiment.

6.5. Metabolic activation system

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

6.5.1. Preparation of S9-fraction

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures. The rats were injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, the rats were sedated using oxygen/carbon dioxide and then killed by decapitation (they were denied access to food for at least 12 hours preceding sacrifice). The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C).

Before use, all S9-batches were characterized with the metabolic activation requiring positive control; benzo[a]pyrene (Sigma) in tester strain TA98 at the concentration of 5 µg/plate.

6.5.2. Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix components contained per 10 ml: 30 mg NADP (Randox) and 15.2 mg glucose-6-phosphate (Roche Diagnostics, Mannheim, Germany) in 5.5 ml or 5.0 ml Milli-Q water (first or second experiment respectively); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment. The S9-batches used were no. 04-9 and 04-10.

6.6. Study design

6.6.1. Dose range finding test

Selection of an adequate range of doses was based on a dose range finding test with strain TA100 and the WP_2uvrA strain, both with and without S9-mix. Eight concentrations, 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate were tested in triplicate. This dose range finding test was reported as a part of the first experiment of the mutation assay. The highest concentration of FR-1435X used in the subsequent mutation assay was the level at which the test substance exhibited limited solubility.

6.6.2. Mutation assay

At least five different doses (increasing with approximately half-log steps) of the test substance were tested in triplicate in each strain.

The test substance was tested both in the absence and presence of S9-mix in each strain, in two independent experiments.

Top agar in top agar tubes was molten and heated to 45°C. The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test substance in dimethyl sulfoxide and either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were turned and incubated in the dark at 37 ± 1 °C for 48 h. After this period revertant colonies (histidine independent (His^+) for *Salmonella typhimurium* bacteria and tryptophan independent (Trp^+) for *Escherichia coli*) were counted.

6.6.3. Colony counting

The revertant colonies (histidine independent c.q. tryptophan independent) were counted manually if less than 40 colonies per plate were present. If more than 40 colonies were present, these could be counted automatically with a Protos model 50000-colony counter. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

6.7. Interpretation

6.7.1. Acceptability of the assay

A *Salmonella typhimurium* reverse mutation assay and/or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory historical range for each tester strain.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D.
TA1535	- S9-mix	3	24	11 ± 11
	+ S9-mix	3	28	10 ± 11
TA1537	- S9-mix	3	18	6 ± 8
	+ S9-mix	3	20	7 ± 9
TA98	- S9-mix	12	41	18 ± 14
	+ S9-mix	12	49	22 ± 18
TA100	- S9-mix	61	195	132 ± 69
	+ S9-mix	59	188	116 ± 80
WP_2uvrA	- S9-mix	4	32	13 ± 15
	+ S9-mix	4	31	13 ± 15

b) The positive control chemicals should produce responses in all tester strains, which are within the laboratory historical range documented for each positive control substance. Furthermore, the mean plate count should be at least three times the concurrent vehicle control group mean.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D.
TA1535	- S9-mix	90	1801	644 ± 894
	+ S9-mix	58	1015	157 ± 267
TA1537	- S9-mix	77	1384	350 ± 600
	+ S9-mix	58	1009	242 ± 487
TA98	- S9-mix	101	1794	528 ± 858
	+ S9-mix	147	1591	578 ± 777
TA100	- S9-mix	320	2099	940 ± 599
	+ S9-mix	197	2227	787 ± 984
WP_2uvrA	- S9-mix	64	1406	700 ± 793
	+ S9-mix	56	1053	200 ± 405

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

6.7.2. Data evaluation and statistical procedures

No formal hypothesis testing was done.
A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants in any tester strain at any concentration is not greater than two times the solvent control value, with or without metabolic activation.
b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:

a) It induces at least a 2-fold, dose related increase in the number of revertants with respect to the number induced by the solvent control in any of the tester strains, either with or without metabolic activation. However, any mean plate count of less than 20 is considered to be not biologically relevant.
b) The positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

6.8. List of protocol deviations

1. The mean plate count of TA1535 in the absence of S9-mix (first experiment; positive control) was not within the laboratory historical range.
   Evaluation: This value was above the upper limit of the range and more than three times the concurrent vehicle control group.

The study integrity was not adversely affected by the deviation.

7. RESULTS

7.1. Dose range finding test

FR-1435X was tested in the tester strains TA100 and WP_2uvrA with concentrations of 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate in the absence and presence of S9-mix.

This dose range finding test is reported as a part of the first experiment of the mutation test (Table 1). The individual data are presented in Appendix II.

Precipitate
The test substance precipitated in the top agar at concentrations of 333 µg/plate and upwards. Precipitation of FR-1435X on the plates was observed at the start of the incubation period at concentrations of 1000 µg/plate and upwards and at concentrations of 3330 and 5000 µg/plate at the end of the incubation period.

Toxicity
To determine the toxicity of FR-1435X, the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined. The definitions are stated in Appendix I.

No reduction of the bacterial background lawn and no biologically relevant decrease in the number of revertants were observed.

Mutagenicity
In the dose range finding test, no increase in the number of revertants was observed upon treatment with FR-1435X under all conditions tested.

7.2. Mutation assay

Based on the results of the dose range finding test, FR-1435X was tested up to concentrations of 3330 µg/plate in the absence and presence of S9-mix in two mutation assays. The first mutation experiment was performed with the strains TA1535, TA1537 and TA98 and the second mutation experiment was performed with the strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. The results are shown in Table 1 and 2, the individual data are presented in Appendix II.

Precipitate
FR-1435X precipitated in the top agar at concentrations of 333 µg/plate and upwards. Precipitation of FR-1435X on the plates was observed at the start of the incubation period at concentrations of 1000 and 3330 µg/plate and at the concentration of 3330 µg/plate at the end of the incubation period.

Toxicity
In both mutation assays, there was no reduction in the bacterial background lawn and no biologically relevant decrease in the number of revertants at any of the concentrations tested in all tester strains in the absence and presence of S9-mix.

Mutagenicity
In both mutation assays, no increase in the number of revertants was observed upon treatment with FR-1435X under all conditions tested.

8. DISCUSSION AND CONCLUSION

All bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments.

The negative and strain-specific positive control values were within our laboratory historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that FR-1435X is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

9. REFERENCES

1 Leonardo, J.M., Dornfeld, S.S. and Peak, M.J., 1984, Evaluation of E. coli K12 343 \ 13 and derived strains for microbial mutagenicity assays. Mutation Res., 130, 87-95.
2 Ames, B.N., McCann, J. and Yamasaki, E., 1975, Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian* microsome mutagenicity test, Mutation Res., 31, 347-364.
3 Maron, D.M. and Ames, B.N., 1983, Revised methods for the *Salmonella* mutagenicity test, Mutation Res., 113, 173-215. Erratum, 1983, Mutation Res., 113, 533.
4 Green, M.H.L. and Muriel, W.J., 1976, Mutagen testing using Trp^+ reversion in *Escherichia coli*, Mutation Res., 38, 3-32.
5 Vogel, H.J. and Bonner, D.M., 1956, Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem., 218, 97-106.

Table 1 **Experiment 1: Mutagenic response of FR-1435X in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance:
TA100 and WP_2uvrA: 22 October 2004
TA1535, TA1537 and TA98: 28 October 2004

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
	Without S9-mix				
positive control	1862 ± 26	318 ± 94	1380 ± 49	878 ± 89	409 ± 29
solvent control	18 ± 3	9 ± 1	14 ± 2	122 ± 14	12 ± 1
3				124 ± 16	10 ± 0
10				124 ± 4	14 ± 3
33	20 ± 2	6 ± 2	13 ± 1	142 ± 14	11 ± 4
100	18 ± 6	8 ± 4	15 ± 4	142 ± 19	11 ± 3
333	20 ± 1	6 ± 4	17 ± 3	150 ± 22	14 ± 0
1000	25 ± 4	5 ± 2	16 ± 3	132 ± 17	16 ± 4
3330 SP	24 ± 1	6 ± 4	16 ± 2	115 ± 13	10 ± 2
5000 SP				127 ± 6	9 ± 1
	With S9-mix[1]				
positive control	312 ± 17	238 ± 6	836 ± 36	750 ± 176	385 ± 19
solvent control	20 ± 7	5 ± 1	20 ± 5	127 ± 5	9 ± 3
3				136 ± 5	9 ± 2
10				130 ± 6	15 ± 4
33	19 ± 3	6 ± 3	22 ± 3	131 ± 18	10 ± 2
100	18 ± 3	7 ± 3	17 ± 6	120 ± 17	12 ± 4
333	15 ± 4	7 ± 2	18 ± 3	138 ± 25	12 ± 4
1000	15 ± 2	7 ± 5	20 ± 4	135 ± 7	9 ± 3
3330 SP	17 ± 2	8 ± 4	16 ± 4	110 ± 8	13 ± 3
5000 SP				126 ± 6	13 ± 2

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
SP Slight Precipitate

Table 2 **Experiment 2: Mutagenic response of FR-1435X in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 5 November 2004

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
Without S9-mix					
positive control	1164 ± 27	377 ± 32	974 ± 47	877 ± 39	638 ± 84
solvent control	9 ± 4	8 ± 3	18 ± 2	138 ± 4	10 ± 2
33	10 ± 2	5 ± 2	11 ± 6	122 ± 23	10 ± 1
100	8 ± 1	4 ± 1	16 ± 2	144 ± 21	13 ± 0
333	10 ± 6	5 ± 2	15 ± 2	133 ± 15	16 ± 2
1000	12 ± 3	6 ± 2	12 ± 3	134 ± 11	11 ± 6
3330 SP	9 ± 3	4 ± 2	14 ± 2	119 ± 6	13 ± 4
With S9-mix					
positive control	117 ± 18	135 ± 33	227 ± 11	580 ± 52	88 ± 13
solvent control	20 ± 4	14 ± 2	33 ± 13	116 ± 15	14 ± 1
33	23 ± 2	22 ± 5	30 ± 3	104 ± 4	12 ± 5
100	18 ± 1	22 ± 5	32 ± 9	114 ± 13	14 ± 2
333	9 ± 2	12 ± 6	28 ± 1	128 ± 21	12 ± 1
1000	23 ± 3	15 ± 2	30 ± 4	117 ± 8	13 ± 3
3330 SP	22 ± 5	24 ± 4	25 ± 6	101 ± 9	18 ± 1

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 10% (v/v) S9 fraction
SP Slight Precipitate

APPENDIX I SUPPORTING MATERIALS AND METHOD

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a slight thinning of the microcolony lawn.
Moderately reduced	Distinguished by a moderate thinning of the microcolony lawn.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:
A reduction of 21-40%: slight reduction.
A reduction of 41-60%: moderate reduction.
A reduction of 61-99%: extreme reduction.
If the size of the microcolonies was increased to small colonies due to an extremely reduced background lawn the reduction is evaluated as microcolonies. If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.
However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

APPENDIX II DETAILED TABLES

Individual plate counts; (following pages)

Experiment 1
Strain TA1535

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		1833	1872	1881	1862 ± 26
solvent control		16	21	18	18 ± 3
33		21	18	22	20 ± 2
100		11	20	22	18 ± 6
333		21	19	19	20 ± 1
1000		21	28	27	25 ± 4
3330	SP	24	23	25	24 ± 1

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		292	320	324	312 ± 17
solvent control		23	12	24	20 ± 7
33		21	21	16	19 ± 3
100		16	21	16	18 ± 3
333		18	10	16	15 ± 4
1000		13	16	16	15 ± 2
3330	SP	18	18	14	17 ± 2

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 1
Strain TA1537

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		241	423	289	318 ± 94
solvent control		8	9	9	9 ± 1
33		5	8	6	6 ± 2
100		6	12	5	8 ± 4
333		9	6	2	6 ± 4
1000		5	4	7	5 ± 2
3330	SP	11	3	4	6 ± 4

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		241	241	231	238 ± 6
solvent control		5	4	5	5 ± 1
33		3	9	7	6 ± 3
100		6	11	5	7 ± 3
333		8	7	5	7 ± 2
1000		12	6	2	7 ± 5
3330	SP	8	5	12	8 ± 4

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 1
Strain TA98

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		1323	1409	1408	1380 ± 49
solvent control		14	12	15	14 ± 2
33		14	13	13	13 ± 1
100		19	12	15	15 ± 4
333		20	16	14	17 ± 3
1000		16	13	19	16 ± 3
3330	SP	16	17	14	16 ± 2

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		875	828	804	836 ± 36
solvent control		26	16	19	20 ± 5
33		19	24	23	22 ± 3
100		12	23	17	17 ± 6
333		15	21	18	18 ± 3
1000		25	18	18	20 ± 4
3330	SP	16	20	13	16 ± 4

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 1
Strain TA100

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		775	932	926	878 ± 89
solvent control		132	127	106	122 ± 14
3		139	126	108	124 ± 16
10		129	122	121	124 ± 4
33		140	157	129	142 ± 14
100		121	158	148	142 ± 19
333		152	128	171	150 ± 22
1000		112	144	139	132 ± 17
3330	SP	106	110	130	115 ± 13
5000	SP	121	127	133	127 ± 6

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		606	698	947	750 ± 176
solvent control		123	132	125	127 ± 5
3		141	136	131	136 ± 5
10		136	128	125	130 ± 6
33		151	116	125	131 ± 18
100		112	139	108	120 ± 17
333		140	161	112	138 ± 25
1000		131	132	143	135 ± 7
3330	SP	119	108	103	110 ± 8
5000	SP	121	126	132	126 ± 6

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 1
Strain WP_2uvrA

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		407	439	381	409 ± 29
solvent control		13	11	12	12 ± 1
3		10	10	10	10 ± 0
10		11	16	15	14 ± 3
33		14	7	12	11 ± 4
100		14	11	9	11 ± 3
333		14	14	14	14 ± 0
1000		14	20	13	16 ± 4
3330	SP	11	11	7	10 ± 2
5000	SP	10	10	8	9 ± 1

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		400	391	363	385 ± 19
solvent control		10	11	6	9 ± 3
3		7	8	11	9 ± 2
10		13	20	13	15 ± 4
33		8	11	12	10 ± 2
100		16	13	8	12 ± 4
333		11	9	16	12 ± 4
1000		12	6	8	9 ± 3
3330	SP	14	10	15	13 ± 3
5000	SP	11	14	15	13 ± 2

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 2
Strain TA1535

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		1134	1174	1185	1164 ± 27
solvent control		10	12	4	9 ± 4
33		9	9	13	10 ± 2
100		8	9	8	8 ± 1
333		5	16	10	10 ± 6
1000		15	12	10	12 ± 3
3330	SP	7	9	12	9 ± 3

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		115	101	136	117 ± 18
solvent control		23	15	21	20 ± 4
33		23	22	25	23 ± 2
100		19	17	17	18 ± 1
333		9	8	11	9 ± 2
1000		20	23	26	23 ± 3
3330	SP	17	26	24	22 ± 5

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 2
Strain TA1537

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		341	402	389	377 ± 32
solvent control		8	6	11	8 ± 3
33		4	8	4	5 ± 2
100		5	5	3	4 ± 1
333		7	6	3	5 ± 2
1000		5	6	8	6 ± 2
3330	SP	2	5	5	4 ± 2

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		173	121	112	135 ± 33
solvent control		12	16	15	14 ± 2
33		18	27	22	22 ± 5
100		21	28	18	22 ± 5
333		8	19	9	12 ± 6
1000		13	15	17	15 ± 2
3330	SP	20	26	27	24 ± 4

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 2
Strain TA98

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		1007	994	920	974 ± 47
solvent control		16	20	19	18 ± 2
33		6	10	18	11 ± 6
100		18	15	15	16 ± 2
333		14	17	15	15 ± 2
1000		15	10	12	12 ± 3
3330	SP	15	12	14	14 ± 2

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		231	215	236	227 ± 11
solvent control		47	22	29	33 ± 13
33		30	28	33	30 ± 3
100		39	21	35	32 ± 9
333		29	27	28	28 ± 1
1000		26	30	33	30 ± 4
3330	SP	24	20	31	25 ± 6

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 2
Strain TA100

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		834	908	890	877 ± 39
solvent control		138	141	134	138 ± 4
33		126	143	97	122 ± 23
100		128	136	167	144 ± 21
333		147	135	118	133 ± 15
1000		141	122	140	134 ± 11
3330	SP	119	113	124	119 ± 6

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		627	589	524	580 ± 52
solvent control		127	121	99	116 ± 15
33		109	102	101	104 ± 4
100		99	123	121	114 ± 13
333		132	147	105	128 ± 21
1000		123	120	108	117 ± 8
3330	SP	112	95	97	101 ± 9

SP: Slight Precipitate

APPENDIX II – continued –

Experiment 2
Strain WP_2uvrA

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		581	599	734	638 ± 84
solvent control		11	10	8	10 ± 2
33		11	9	11	10 ± 1
100		13	13	13	13 ± 0
333		14	18	17	16 ± 2
1000		17	6	11	11 ± 6
3330	SP	17	11	10	13 ± 4

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		75	100	90	88 ± 13
solvent control		15	14	13	14 ± 1
33		16	13	6	12 ± 5
100		15	16	12	14 ± 2
333		11	11	13	12 ± 1
1000		13	10	16	13 ± 3
3330	SP	19	19	17	18 ± 1

SP: Slight Precipitate

FR-1435X

NOTOX Project 419906

APPENDIX III CERTIFICATE OF ANALYSIS

IMI (TAMI) Institute for Research & Development Ltd
Flame Retardants Department
P.O.Box 10140, Haifa Bay 26111, Israel Tel.+972-4-8469553
Fax:+972-4-846-9320 E-mail:zilbermanj@tami-imi.co.il

תמי (אימי) מכון למחקר ולפתוח בעיים
מחלקת עכבי תאורה
ת.ד. 10140, מפרץ חיפה 26111 טל 04-8469553
פקס: 04-8469320 E-mail: zilbermanj@tami-imi.co.il

To: Lilach Yakobovich - DSBG

From: Y. Zilberman - IMI

Date: 14 December 2003

Following are some of the characteristics of the products

CONFIDENTIAL

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APPENDIX IV PROTOCOL

ORIGINAL

PROTOCOL

Study Title

EVALUATION OF THE MUTAGENIC ACTIVITY OF FR-1435X IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY (WITH INDEPENDENT REPEAT)

Author

C.M. Verspeek-Rip

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419906
NOTOX Substance 146232/A

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FR-1435X NOTOX Project 419906

2. PROTOCOL APPROVAL

STUDY DIRECTOR: C.M. Verspeek-Rip

date: 05 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.

date: 5-10-04

SPONSOR: Dead Sea bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

date: 01.10.04

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FR-1435X NOTOX Project 419906

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	C.M. Verspeek-Rip
Technical Coordinator	Ing. A. Tijdink
Study Plan	Start week beginning : 18 October 2004 (week 43) Completed week beginning : 15 November 2004 (week 47) Proposed Reporting date : 12 December 2004

3.2. Aims of study

The objective of this study is to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent *Salmonella typhimurium* strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

Background of the test system
The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay has shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay will be conducted in the absence and in the presence of a metabolic system (S9-mix). The *Salmonella typhimurium* strains used in this study will be TA98, TA100, TA1535 and TA1537, the *Escherichia coli* strain used will be WP_2uvrA. The strains TA98 and TA1537 are capable of detecting frameshift mutagens, the strains TA100, TA1535 and WP_2uvrA are capable of detecting base-pair substitution mutagens (1, 2, 3, 4 and 5).

3.3. Guidelines

The study procedures described in this protocol are based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test". (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria". EEC Publication Commission Directive (Published June 8, 2000).

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3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997)

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections will be also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

[CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	

APPENDIX IV – continued –

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Water	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

The sponsor is responsible for all test substance data unless determined by NOTOX

4.1.2. Test substance preparation

In order to obtain a suitable solvent solubility test will be performed. Standard solvents used for diluting test substances will be, in order of preference, Milli-Q water, dimethyl sulfoxide of spectroscopic quality and ethanol. Other solvents compatible with the test system will be used when necessary. Test substance concentrations will be used within 4 hours after preparation.

Concentrations and vehicle will be approved by the study director in the study files.

4.2. Reference substances

4.2.1. Negative control

The vehicle of the test article.

4.2.2. Positive controls

Without metabolic activation (-S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1535	sodium azide (SA)	5 µg	Saline
TA1537	9-aminoacridine (9AC)	60 µg	Milli-Q water
TA98	2-nitrofluorene (NF)	10 µg	DMSO
TA100	methylmethanesulfonate (MMS)	650 µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO)	10 µg	DMSO

With metabolic activation (+S9-mix):
The positive control substance used for all tester strains will be 2-aminoanthracene (2AA). The following doses will be used:

Strain	Concentration/plate	Amount of S9-mix	Solvent
TA1535	1 µg	5 and 10%	DMSO
TA1537	2.5 µg	5%	DMSO
TA1537	5 µg	10%	DMSO
TA98	1 µg	5 and 10%	DMSO
TA100	1 µg	5%	DMSO
TA100	2.5 µg	10%	DMSO
WP_2uvrA	10 µg	5 and 10%	DMSO

Solvents for reference substances
Saline = physiological saline
DMSO = dimethyl sulfoxide of spectroscopic quality
Milli-Q water

4.3. Test system

Test System *Salmonella typhimurium* bacteria and *Escherichia coli* bacteria

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Rationale	Recommended test system in international guidelines as (e.g. OECD, EEC).
Source	Dr Bruce N. Ames, University of California at Berkeley, U.S.A (TA98: 1991, TA1535 and TA1537: 2001) Xenometric, Boulder, Co, U S A (obtained from N.V. Organon) (TA100: 2002) Prof. Dr B A. Bridges, University of Sussex, Brighton, U K. (1987) (*Escherichia coli* strain)

The characteristics of the different *Salmonella typhimurium* strains are as follows:

Strain	Histidine mutation	Mutation type
TA1537	*his*C3076	Frameshift
TA98	*his*D3052/R-factor*	Frameshift
TA1535	*his*G46	Base-pair substitutions
TA100	*his*G46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contains the following additional mutations.
rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB : loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains are regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The sensitivity of the strain to a wide variety of mutagens has been enhanced by permeabilization of the strain using Tris-EDTA treatment (ref.1). The strain is regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains are stored in liquid nitrogen (-196°C).

4.4. Cell culture

Preparation of bacterial cultures
Samples of frozen stock cultures of bacteria will be transferred into enriched nutrient broth and incubated in a shaking incubator (37°C, 150 spm), until the cultures reach an optical density of 1.0 ± 0.1 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain will be used for a test.

Agar plates
Agar plates (ø 9 cm) contain 25 ml glucose agar medium. Glucose agar medium contains per liter: 18 g purified agar in Vogel-Bonner Medium E, 20 g glucose. N.B. The agar plate for the test with the Salmonella typhimurium strains also contains 12.5 µg/plate biotin and 15 µg/plate histidine and the agar plates for the test with the *Escherichia coli* strain contains 15 µg/plate tryptophan.

Top agar
Top agar medium, containing 0.6% (w/v) bacteriological agar and 0.5% (w/v) Sodium Chloride, will be heated to dissolve the agar. Samples of 3 ml top agar will be transferred into 10 ml glass tubes with metal caps. Top agar tubes will be autoclaved for 20 min at 121 ± 3 °C.

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Environmental conditions
All incubations will be carried out in the dark at 37 ± 1 °C. The temperature will be monitored during the experiment.

4.5. Metabolic activation system

Rat liver microsomal enzymes are routinely prepared from adult male Wistar rats, which are obtained from Charles River (Sulzfeld, Germany).

4.5.1. Preparation of S9-fraction

The animals are housed at NOTOX in a special room under standard laboratory conditions, as described in the SOP's. The rats are injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days after exposure, the rats are sedated using oxygen/carbon dioxide and then killed by decapitation, (they are denied access to food for at least 12 hours preceding sacrifice). The livers of the rats are removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers are minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate is centrifuged for 15 min at 9000 g. The supernatant (S9) is transferred into sterile ampules, which are stored in liquid nitrogen (-196°C).

The S9 batch is characterised with the metabolic requiring mutagens Benzo[a]pyrene in tester strain TA98 at the concentration of 5 µg/plate.

4.5.2. Preparation of S9-mix

S9-mix will be prepared immediately before use and kept on ice. S9-mix components contains per 10 ml; 30 mg NADP and 15.2 mg glucose-6-phosphate in 5.5 ml or 5.0 ml Milli-Q water (first or second experiment respectively); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution will be filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction will be added (5% (v/v) S9-fraction) to complete the S9-mix and to 9.0 ml of S9-mix components 1.0 ml S9-fraction will be added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment.

4.6. Study design

4.6.1. Dose range finding test

Selection of an adequate range of doses is based on a dose range finding test with strain TA100 and the WP_2uvrA strain, both with and without S9-mix. Eight concentrations of the test substance, 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate will be tested in triplicate. This dose range finding test will be reported as the first experiment of the mutation assay. The highest concentration of test substance used in the subsequent mutation assay will be 5 mg/plate or the level at which the test substance inhibits bacterial growth unless the test substance exhibits limited solubility or will be not uniformly dispersible in the solvent of choice.

4.6.2. Mutation assay

At least five different doses (with approximately half-log steps) of the test substance will be tested in triplicate in each strain. The test substance is tested both in the absence and presence of S9-mix in two independent experiments.

Top agar in top agar tubes will be molten and heated to 45°C. The following solutions will be successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test substance in a suitable solvent, and

either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients will be mixed on a Vortex and the content of the top agar tube will be poured onto a selective agar plate. After solidification of the top agar, the plates will be turned and incubated in the dark at 37 ± 1°C for 48 h. After this period revertant colonies (histidine independent for *Salmonella typhimurium* bacteria and tryptophan independent for *Escherichia coli* bacteria) will be counted.

4.6.3. Colony counting

The revertant colonies (histidine independent c.q. tryptophan independent) will be counted manually if less than 40 colonies per plate are present. If more than 40 colonies are present, these can be counted automatically with a Protos model 50000-colony counter. Plates with sufficient test article precipitate to interfere with automated colony counting will be counted manually.

4.7. Interpretation

4.7.1. Acceptability of the assay

A *Salmonella typhimurium* reverse mutation assay and/or or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory historical range for each tester strain.
b) The positive control chemicals should produce responses in all tester strains, which are within the laboratory historical range documented for each positive control substance. Furthermore, the mean plate count should be at least three times the concurrent vehicle control group mean.
c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

4.7.2. Data evaluation and statistical procedures

No formal hypothesis testing will be done.
A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants in any tester strain at any concentration is not greater than two times the solvent control value, with or without metabolic activation.
b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:

a) It induces at least a 2-fold, dose related increase in the number of revertants with respect to the number induced by the solvent control in any of the tester strains, either with or without metabolic activation. However, any mean plate count of less than 20 is considered to be not biologically relevant.
b) In case a positive result will be repeated, the positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria are not absolute and other modifying factors may enter into the final evaluation decision.

APPENDIX IV – continued –

FR-1435X NOTOX Project 419906

5. REFERENCES

1 Leonardo, J.M., Dornfeld, S.S. and Peak, M.J., 1984, Evaluation of *E. coli* K12 343 \ 13 and derived strains for microbial mutagenicity assays. Mutation Res., 130, 87-95.
2 Ames, B.N., McCann, J. and Yamasaki, E., 1975, Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian* microsome mutagenicity test, Mutation Res., 31, 347-364.
3 Maron, D.M. and Ames, B.N., 1983, Revised methods for the *Salmonella* mutagenicity test, Mutation Res., 113, 173-215. Erratum, 1983, Mutation Res., 113, 533.
4 Green, M.H.L. and Muriel, W.J., 1976, Mutagen testing using Trp^+ reversion in *Escherichia coli*, Mutation Res., 38, 3-32.
5 Vogel, H.J. and Bonner, D.M., 1956, Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem., 218, 97-106.

6. DISTRIBUTION

Original: Study Diroctor
1 Copy: Technical Coordinator
1 Copy: QAU/Management
1 Copy: Sponsor

APPENDIX IV – continued –

ORIGINAL

PROTOCOL AMENDMENT NO : 1

Study Title	Evaluation of the mutagenic activity of FR-1435X in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat)
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419906

AMENDMENT DESCRIPTION

1. Sponsor: Dead Sea Bromine Group
Purchasing Division of DSBG
P.O. Box 180
BEER-SHEVA 84101
Israel

REASONS FOR AMENDMENT

1. Typing error.

APPROVAL
Study director

C.M. Verspeek-Rip

date: 19 October 2004

APPENDIX IV – continued –

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title	Evaluation of the mutagenic activity of FR-1435X in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat)
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419906

AMENDMENT DESCRIPTION

1.	Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
2.	Description	Brown liquid

REASONS FOR AMENDMENT

1. Typing error.
2. The data has been updated according to information received from the sponsor dated January 10, 2005.

APPROVAL
Study director

C.M. Verspeek-Rip

10 January 2005
date:

- Attachment 14 -

IMI (TAMI)
Institute for R&D Ltd.
Member of ICL Group

(אימי) תמי
מכון למחקר ולפתוח בע"מ
בקבוצת כימיקלים לישראל

READY BIODEGRADABILITY TEST OF FR-1435X AND FR-1335X: CO_2 IN SEALED VESSELS (HEADSPACE TEST)

FINAL REPORT

AUTHOR:
Smadar Admon

STUDY INITIATION DATE: 10 December 2003

STUDY COMPLETION DATE: 6 January 2004

PERFORMING LABORATORY:
Water and Wastewater Laboratory
IMI (TAMI) Institute of Research & Development Ltd.
P.O.Box. 10140, Haifa Bay 26111, Israel

LABORATORY PROJECT NUMBER: 103247

Submitted to
Dead Sea Bromine Group / Bromine Compounds Ltd.
P.O.Box 180, Beer Sheva, 84101, Israel

February 2004

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

10-d window	= The 10 days following the attainment of 10% biodegradation
FR	= Flame retardant
FR-1435X	= Benzene, pentabromo[[2-(2-methoxymethylethoxy) methylethoxy] methyl]
FR-1335X	= Ethanol, methyl-2-[methyl-2-[(pentabromophenyl) methoxy] ethoxy]
GC	= Gas chromatograph
IC	= Inorganic carbon
MLSS	= Mixed liquor suspended solids
PBT	= Persistence, Bioaccumulation and Toxicity
ThIC	= Theoretical inorganic carbon
TIC	= Total inorganic carbon
WWTP	= Wastewater treatment plant

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

REPORT APPROVAL

Study Director:

IMI Water and Wastewater Laboratory

By: _____ 10/2/04

Smadar Admon, Ph.D (Date)

Report approval / Issued:

IMI Water and Wastewater Laboratory

By: Dorit 12/2/04

Dorit Abramovich , Ph.D (Date)

Conducted:

IMI Water and Wastewater Laboratory

_____ 10.2.2004

Mariana Friedman (Date)

SUMMARY

The present study was designed to assess the biodegradation potential of the two flame retardants FR-1435X and FR-1335X by the 'Ready biodegradability test', in which the biodegradation of the chemicals is tested in reactions where the microorganisms concentration is low while the concentration of the test substances is relatively high. The test was conducted according to the method 'CO_2 in sealed vessels (headspace test)' under the proposed new guideline by the Organization for Economic Cooperation and Development (OECD 310) and the US EPA OPPTS guideline 835.3120. The biodegradation of the chemicals is determined by measuring the CO_2 evolved by the ultimate biodegradation (mineralization) of the test compounds. Since both FR-1335X and FR-1435X have poor solubility in water, they had to be dissolved first in acetone prior to dispensing on GF/A filters, which were incubated with inoculated medium. The reference compound used was n-octanol. A chemical is classified as readily biodegradable if more than 60% of its carbon content was released as CO_2 during the 28 days of the test.

The results from the biodegradability test indicate that the two substances are not readily biodegradable. The test was proven valid since n-octanol was mineralized by an average 89% during the test period. In addition, FR-1435X and FR-1335X were not inhibitory to the biomass at the test conditions.

1. INTRODUCTION

The present study was designed to assess the biodegradation potential of the two flame retardants FR-1435X and FR-1335X by the 'Ready biodegradability test'. Since these compounds have poor water solubility, the method of choice for testing the biodegradation has to be based on respirometric methods where production of CO_2 or consumption of O_2 during ultimate biodegradation are measured. Therefore, the present test was conducted according to the method 'CO_2 in sealed vessels (headspace test)' under the proposed new guideline by the Organization for Economic Cooperation and Development (OECD 310) and the US EPA OPPTS guideline 835.3120. The protocol for the test is attached as appendix 4 to this report.

The test substance, at approximately 20mgC/L, as the sole source of carbon and energy, was incubated in a buffer-mineral salts medium inoculated with a mixed population of microorganisms originating from a municipal wastewater treatment plant (WWTP). Due to the low solubility of the test FRs, the compounds were first dissolved in acetone, dispensed on glass fiber filters and then introduced into the reaction mixture following the evaporation of the solvent. The test was performed in sealed bottles with a headspace of air, which provided a reservoir of oxygen for aerobic biodegradation. CO_2 evolution, resulting from the ultimate aerobic biodegradation of the test substance, was determined in the test bottles in excess of the CO_2 produced in blank vessels containing inoculated medium only. The values were translated to equivalent inorganic carbon (IC) produced and the extent of biodegradation was expressed as the percentage of the maximum theoretical IC (ThIC) production, based on the quantity of test substance (as organic carbon) added initially. The reference compound used as the procedure control was n-octanol (CAS 111-87-5), a water insoluble substance known to be biodegradable. A chemical is defined as ready biodegradable if >60% of its ThIC content is biodegraded within a '10-d window' during the 28 days of the test period.

2. OBJECTIVE

The objective of this study was to evaluate the ready biodegradability of the two poorly water soluble flame retardants FR-1435X and FR-1335X. The test was based on the proposed new guideline by the Organization for Economic Cooperation and Development (OECD 310) and on the US EPA OPPTS guideline 835.3120. The test, CO_2 in sealed vessels (headspace test), is suitable for testing insoluble substances.

3. MATERIALS AND METHODS

3.1 General

Reagent chemicals were used in all tests. Distilled water was used for dilutions.

3.2 Test substances

The two substances tested were:

[ ] CONFIDENTIA

Certificates of analysis for the two test substances were provided by sponsor and attached as appendices 2 and 3.

The concentrations used in the test were equivalent to 20mgC/L, i.e.75.5 mg/L for FR-1435X and 82 mg/L for FR-1335X. Following are the specifications of the substances:

[ ]

CONFIDENTIAL

3.3 Reference substance

The reference substance was n-octanol ($C_8H_{18}O$), purchased from Fluka (74850).

The concentration used in the test was 27 mg/L, equivalent to 20 mgC/L

3.4 Growth media

The mineral medium used for the experiment consisted of the following compounds: 85 mg/L KH_2PO_4, 220 mg/L K_2HPO_4, 330 mg/L $Na_2HPO_4{\cdot}2H_2O$, 5 mg/L NH_4Cl, 36 mg/L $CaCl_2{\cdot}2H_2O$, 22.5 mg/L $MgSO_4{\cdot}2H_2O$, 15 mg/L $FeCl_3$ at pH 7.4.

3.5 CO_2 free water

CO_2 free water was obtained by purging water with CO_2 free air at pH 6.5 during the night before the initiation of experiment. CO_2 free air was prepared by passing ambient air through a column containing soda lime pellets with indicator (Merck 106839).

3.6 Inoculum

The inoculum used for the test consisted of activated sludge from Haifa municipal WWTP. The sludge was collected two days prior to the initiation of the experiment and aerated during two days in order to reduce carbonaceous background. The day before the experiment was started, the sludge was diluted in the experimental medium to yield a final MLSS concentration of 100 mg/L and then continued to be aerated during the night with CO_2 free air.

3.7 Ready Biodegradability Test

The biodegradability test was conducted according to the protocol (Appendix 4). The test substances FR-1435X and FR-1335X were dissolved in acetone and then 50µl were dispensed on GF/A filters giving a final nominal concentration of 20mgC/L. The filters were weighed before the addition of the substances and after the evaporation of the acetone in order to measure the initial amount of test substances added to each reaction bottle and calculate the ThIC. After evaporation of the acetone, the filters were placed in 160 ml serum bottles, 100 ml of inoculated medium were added under continuous flushing of CO_2 free air and then the bottles were sealed with butyl rubber stopper and aluminum crimps. The reference substance n-octanol was dispensed directly on the filters.

The test contained a few controls (Table 2): A blank control where clean GF/A filters were introduced into the pre-inoculated medium (A), a procedure control where the biodegradation of n-octanol was measured (B), inhibition controls where the effects of the test substances on n-octanol biodegradation were estimated (D and G), and abiotic controls where the inoculum

was poisoned with $HgCl_2$ (E and H). The biodegradation process was followed by periodical measurements of the CO_2 evolution. At each time point (Table 3), three bottles were sacrificed by acidification and then the CO_2 in the head space was measures using Gas chromatography (GC).

Table 2: Ready biodegradability test set up

(X) – present in reaction mixture, (-) – excluded from reaction mixture

Reaction mixture	Sample ID	FR-1435X 20mgC/L	FR-1335X 20mgC/L	n-octanol 20mg/L	$HgCl_2$ (mg/L)	Mineral medium (ml)
Blank	A1 – A29	-	-	-	-	Up to 100 ml
Procedure control	B1 – B20	-	-	X	-	Up to 100 ml
FR-1435X -Test	C1 – C32	X	-	-	-	Up to 100 ml
FR-1435X - Inhibition	D1 – D6	X	-	X	-	Up to 100 ml
FR-1435X - Abiotic	E1 – E6	X	-	-	X	Up to 100 ml
FR-1335X - Test	F1 – F32	-	X	-	-	Up to 100 ml
FR-1335X - Inhibition	G1 – G6	-	X	X	-	Up to 100 ml
FR-1335X - Abiotic	H1 – H6	-	X	-	X	Up to 100 ml

Table 3: Sampling schedule

Number of bottles analyzed

Day	1	5	9	16	21	28
Blank	3	3	3	3	3	5
Procedure control	3	3	3	3	3	5
FR-1435X -Test	3	3	3	3	3	5
FR-1435X - Inhibition	3	-	-	-	-	3
FR-1435X - Abiotic	3	-	-	-	-	3
FR-1335X - Test	3	3	3	3	3	5
FR-1335X - Inhibition	3	-	-	-	-	3
FR-1335X - Abiotic	3	-	-	-	-	3

3.8 Analytical methods

Each bottle to be analyzed was acidified by injecting 1 ml of concentrate H_3PO_4 (85%) through the septum using a syringe and a 20 G needle. The bottle was shaken for at least an hour at the experimental temperature (21°C) in order to reach liquid/gas equilibrium. From each analyzed bottle, an 1 ml aliquot of the headspace was withdrawn using a gas tight syringe and injected into in a GC equipped with a thermal conductivity detector for CO_2 determination.

4. RESULTS

The results from the biodegradability test are presented in tables 4 and 5. In the samples containing either FR-1435X or FR-1335X, no CO_2 was produced during the 28 days and therefore ultimate biodegradation did not occur. In the procedure control, n-octanol was removed by 89% during the test period, indicating that the test was valid and the inoculum was viable (Figure 1). In the inhibition controls, CO_2 evolution was equivalent to a degradation extent of 45% and 42% of ThIC introduced, indicating that the test substances were not inhibitory to the inoculum at the concentrations used. Assuming that the CO_2 originated solely from the degradation of n-octanol, the n-octanol degradation was equivalent 92% and 85% in the presence of FR-1435X and FR-1335X, respectively (Table5).

Table 4: Inorganic carbon evolution

ThIC is theoretical inorganic carbon, IC is inorganic carbon, sd is standard deviation, ND is not determined.
[a] – ThIC concentrations are calculated from average net weight of substances dosed on all filters (Appendix 1). [b] – Average production of CO_2 from three or five sacrificed bottles (Table 3) was translated to IC according to calculations in appendix 1.

Reaction mixture	Initial ThIC[a] (mg/L)	IC produced [b] (mg/L)				
		day 5	day 9	day 16	day 21	day 28
Blank	0	0.43 (sd = ± 0.04)	0.3 (sd = ± 0.02)	0.47 (sd = ± 0.04)	0.64 (sd = ± 0.05)	0.74 (sd = ± 0.09)
Procedure control	21.6 (sd = ± 0.37)	13.07 (sd = ± 0.61)	16.87 (sd = ± 0.65)	17.43 (sd = ± 1.04)	19.70 (sd = ± 0.36)	19.9 (sd = ± 1.17)
FR-1435X -Test	21.8 (sd = ± 0.38)	0.3 (sd = ± 0.40)	0.35 (sd = ± 0.09)	0.36 (sd = ± 0.05)	0.64 (sd = ± 0.16)	0.64 (sd = ± 0.09)
FR-1435X - Inhibition	42.9 (sd = ± 0.16)	ND	ND	ND	ND	19.47 (sd = ± 0.46)
FR-1435X - Abiotic	21 (sd = ± 0.75)	ND	ND	ND	ND	0.03 (sd = ± 0.03)
FR-1335X - Test	22.2 (sd = ± 0.56)	0.32 (sd = ± 0.04)	0.34 (sd = ± 0.14)	0.17 (sd = ± 0.07)	0.52 (sd = ± 0.07)	0.66 (sd = ± 0.11)
FR-1335X - Inhibition	44 (sd = ± 0.29)	ND	ND	ND	ND	18.60 (sd = ± 2)
FR-1335X - Abiotic	22.4 (sd = ± 0.95)	ND	ND	ND	ND	0.1 (sd = ± 0.17)

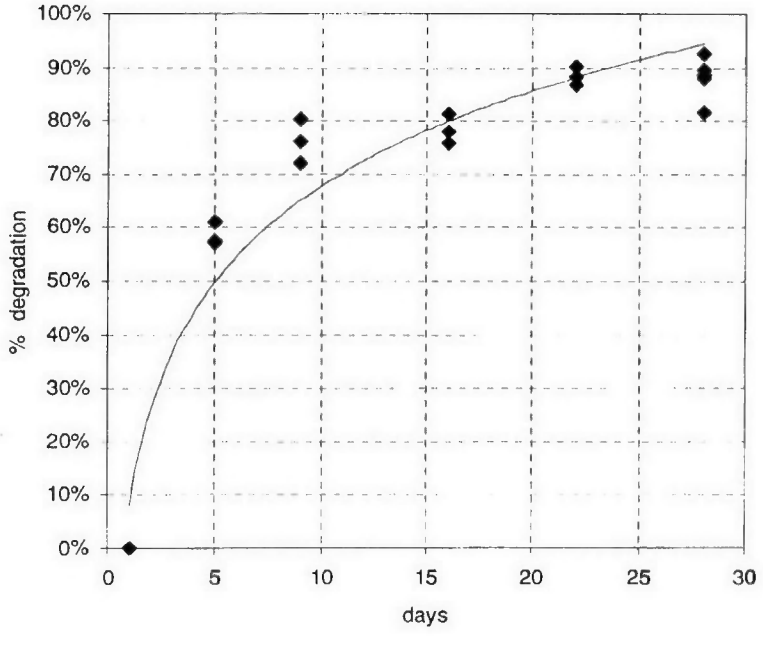
Table 5: % Calculated biodegradation after 28 days

[a] - Average degradation calculated according to appendix 1.
[b] - Average degradation of ThIC including the test substance and n-octanol
[c] - Average degradation of n-octanol in inhibition controls.
NA – not applicable

Reaction mixture	% degradation [a]	% n-octanol degradation [c]
Procedure control	89	NA
FR-1435X -Test	1	NA
FR-1435X - Inhibition [b]	45	92
FR-1435X - Abiotic	0	NA
FR-1335X - Test	0	NA
FR-1335X - Inhibition [b]	42	85
FR-1335X - Abiotic	0	NA

Figure 1: Procedure control – n- octanol biodegradation

Each point represents a sacrificed bottle. Percentage of degradation was calculated after subtraction of background blank control (appendix 1).



5. SUMMARY AND CONCLUSIONS

The aim of the present study was to assess the ready biodegradability of two substances, FR-1435X and FR-1335X, that have very low water solubility. In ready biodegradability tests, the ultimate biodegradation of compounds is determined after a period of 28 days using relatively low concentrations of microorganisms and high concentrations of substances (20mgC/L, equivalent to ~80mg/L of present study substances). When analyzing insoluble substances, the way to follow mineralization is by measuring carbon dioxide evolution. The protocol used for the test (proposed OECD 310) was based on measurement of CO_2 produced during ultimate biodegradation.

The results from the biodegradability test indicate that the two substances are not readily biodegradable. The test was proven valid since n-octanol was mineralized by an average 89% during the test period. In addition, FR-1435X and FR-1335X were not inhibitory to the biomass at the test conditions.

6. REFERENCES

1. Organization for Economic Cooperation and Development (OECD) (October 2003): Proposal for a new guideline 310 for Testing of Chemicals: Ready biodegradability – CO_2 in sealed vessels (Headspace test).
2. US EPA (January 1998): Fate, Transport and Transformation Test Guidelines: OPPTS 825.3120 Sealed-Vessels CO_2 Production Test.

APPENDIX 1: CALCULATIONS

1. ThIC calculation

The Theoretical Inorganic Carbon (ThIC) is the maximal amount of inorganic carbon expected to be generated as CO_2. This amount is equal to the amount of carbon present in the compound. The amount of carbon in the different tested substances is as follows:

Substance	Fraction of C
FR-1435X	0.265
FR-1335X	0.252
n-octanol	0.74

The concentration of initial ThIC (mg/L) is:

$$(Wi * Cf) / V$$

where

Wi = initial weight of substance applied on filter (mg)

Cf = Fraction of carbon in molecule (w/w)

V = Volume of reaction medium (L)

2. IC calculation

The inorganic carbon produced during the ultimate biodegradation is measured as CO_2. Since the carbon fraction in CO_2 is 0.27, the value of CO_2 measured (mg/L) is multiplied by 0.27 in order to obtain the equivalent carbon value.

3. Percent degradation

The percent degradation is the percentage of C produced as CO_2 out of the initial ThIC.

$$\% \text{ degradation of test substance} = (C_t - C_b) / \text{ThIC}$$

where

C_t = Carbon produced as CO_2 at time t in test sample (mg/L)

C_b = Carbon produced as CO_2 at time t in blank control(mg/L)

ThIC = Theoretical inorganic carbon introduced as the test chemical (mg/L)

APPENDIX 2: Certificate of analysis

CONFIDENTIAL

Batch No.	38278-51-1
Appearance, Color	Amber liquid
Assay, % (HPLC area) Impurities	97.5 2.5
Water, ppm	<500
Br total, %	63
Bromides, ppm	<200
Density, kg/m^3 at 25°C	~2000
Viscosity, cps at 23°C	1620
Solubility, g/100 g Water Toluene	 <0.1 complete

APPENDIX 3: Certificate of analysis

CONFIDENTIAL

Batch No.	38278-43-4
Appearance, Color	Yellowish wax
Assay, % (HPLC area) Impurities	96 4
Water, ppm	<500
Br total, %	64.6
Bromides, ppm	<100
Density, kg/m^3 at $25^{\circ}C$	~2000
Melting point, $^{\circ}C$	~40-45
Solubility, g/100 g Water Toluene	 <0.1 complete

APPENDIX 4: PROTOCOL

READY BIODEGRADABILITY TEST OF **FR-1435X AND FR-1335X:** CO_2 IN SEALED VESSELS (HEADSPACE TEST)

Sponsor:
Bromine compounds Ltd. (BCL)
P.O.Box 180
Ltd.
Maklef house
Beer Sheva 84101
Israel

Research Laboratory:
IMI (TAMI)
Institute for Research & Development
P.O.Box 10140
Haifa Bay 26111
Israel

IMI study number: 103247

IMI (TAMI)
Institute for R&D Ltd.
Member of ICL Group

(אימי) תמי
מכון למחקר ולפתוח בע"מ
בקבוצת כימיקלים לישראל

PROTOCOL APPROVAL

READY BIODEGRADABILITY TEST OF **FR-1435 AND FR-1435**
CO_2 IN SEALED VESSELS (HEADSPACE TEST)

_____ _____

Dr. Smadar Admon — Date
Study director
IMI(TAMI) Wastewater Treatment Laboratory

_____ _____

Dr. Orit Manor — Date
Sponsor's monitor
Dead Sea Bromine Group Ltd.

The signature of the study director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the study director's signature will be documented in an amendment.

Proposed experimental dates : December 2003 – February 2004

TABLE OF CONTENTS

ABBREVIATIONS

10-d window = the 10 days following the attainment of 10% biodegradation

GC	= Gas chromatograph
IC	= Inorganic carbon
ThIC	= Theoretical inorganic carbon
TIC	= Total inorganic carbon

1. OBJECTIVE

The objective of this study is to evaluate the ready biodegradability of the two poorly water soluble flame retardants FR-1435X and FR-1335X. The test will be based on the proposed new guideline by the Organization for Economic Cooperation and Development (OECD 310) and on the US EPA OPPTS guideline 835.3120. The test, CO_2 in sealed vessels (headspace test), is suitable for testing insoluble substances.

2. PRINCIPLE OF THE TEST

When testing for the ultimate biodegradability of insoluble substances, the methods of choice are the respirometric methods where CO_2 evolution or O_2 consumption are measured. The present test is based on measurement of CO_2 produced during the ultimate biodegradation of the tested substances. The test substance, at approximately 20mgC/L, as the sole source of carbon and energy, is incubated in a buffer-mineral salts medium which has been inoculated with a mixed population of microorganisms. The test is performed in sealed bottles with a headspace of air, which provides a reservoir of oxygen for aerobic biodegradation. The CO_2 evolution, resulting from the ultimate aerobic biodegradation of the test substance, is determined in the test bottles in excess of the CO_2 produced in the blank vessels containing inoculated medium only. The CO_2 values are translated to equivalent inorganic carbon (IC) produced and the extent of biodegradation is expressed as the percentage of the maximum theoretical IC (ThIC) production, based on the quantity of test substance (as organic carbon) added initially. A few controls are run in parallel: (1) Blank controls, containing inoculum and mineral medium, but no test compound , (2) Procedure control , containing a reference biodegradable substance (1-octanol) and, (3) Inhibition control, containing both the test and reference substances, in order to check possible inhibition to the microorganisms.

The test is normally run for a period of 28 days but may be prolonged if degradation has begun by day 28. Biodegradation of > 60% ThIC within a '10-d window' (the 10 days following the attainment of 10% biodegradation) in the test, demonstrates that the test substance is readily biodegradable under aerobic conditions.

3. MATERIALS AND METHODS

3.1 Reagents

Analytical grade reagents will be used .

3.2 Test substances

The two substances to be tested are:

FR-1435 : Benzene, pentabromo[[2-(2-methoxymethylethoxy) methylethoxy] methyl] FR-1335: Ethanol, methyl-2-[methyl-2-[(pentabromophenyl) methoxy] ethoxy]

FR-1435 and FR-1335 and all the available information on the substances will be supplied by IMI.

3.3 Reference substance

In order to check the functional capability of the activated sludge and the possible inhibitory effect of FR-1435 and FR-1335, tests with a reference substance will be run in parallel. The reference substance that will be used is 1-Octanol, CAS # 111-87-5, which is also insoluble in water.

3.4 Water

Water for dilutions will be prepared by sparging overnight distilled water at pH 6.5 with CO_2 free air. The CO_2 free air will be prepared by passing air through soda lime pellets.

3.5 Mineral medium

Mineral medium will be prepared from the stock solutions (a), (b), (c), (d) as described in appendix 8.1.

3.6 Inoculum

The inoculum will consist of activated sludge collected at a Haifa Municipal Wastewater Treatment Plant. In order to reduce CO_2 background levels, the sludge will be aerated for a few days (no longer than 7 days) before using it for the test.

4. TEST PROCEDURE

4.1 Apparatus

(a) Glass serum bottles, sealed with butyl rubber stoppers and crimp-on aluminum seals. The total volume of each bottle is 160 ml.

(b) Gas chromatograph for measuring inorganic carbon in headspace (CO_2).

(c) Syringes of high precision for gaseous and liquid samples.

(d) Glass filters of grade GF/A as support for the substances.

(e) A supply of CO_2 free air prepared by passing air through a column of soda lime pellets.

(f) Orbital shakers in a temperature controlled environment (20-25°C).

4.2 Test substances

The test substances will be dissolved in acetone prior to dispensing on a 21mm GF/A filter. 50 µl of the stock solution of each substance will be dispensed on each filter and after evaporation of the acetone the filter will be introduced to the reaction bottle. The amount of substance on each filter will be equivalent to 20 mgC/L.

4.3 Reference substance

1-octanol will be introduced to test reactions following direct dispensing on 21mm GF/A filter at a quantity equivalent to a concentration of 20mgC/L.

4.4 Inoculated medium

4.4.1 Preconditioning of the inoculum

In order to reduce CO_2 background levels, the inoculum will be preconditioned to the experimental conditions by aerating the sludge after diluting it in test medium to 50-100 mg/L with CO_2 free moist air overnight.

4.4.2 Inoculated medium

The inoculum will be used at a final concentration of 4-10 mg/L activated sludge dry solids. Immediately before use, the inoculated medium will be prepared by adding the appropriate volume of the preconditioned activated sludge to the mineral medium.

4.5 Test procedure

4.5.1 Bottles preparation

Aliquots of 100 ml inoculated medium will be dispensed into replicate bottles of 160-ml capacity, leaving a headspace of 40 ml. The number of bottles in each set will be such that at each sampling time, three bottles can be sacrificed. The test substance will be introduced on a filter as described in 4.3. The following sets will be prepared:

(A) Blank controls containing clean GF/A filters.
(B) Procedure controls containing reference substance (1-octanol) on GF/A filters.
(C) FR-1435 test vessels containing the test substance FR-1435X on GF/A filters.
(D) Inhibition controls containing FR-1435X and 1-octanol on GF/A filters.
(E) Abiotic control vessels containing FR-1435X and 50mg/L $HgCl_2$.
(F) FR-1335 test vessels containing the test substance FR-1335X on GF/A filters.
(G) Inhibition controls containing FR-1335X and 1-octanol on GF/A filters.
(H) Abiotic control vessels containing FR-1335X and 50mg/L $HgCl_2$.

All bottles will be sealed with butyl rubber septa and aluminum crimps.

4.5.2 Incubation

The sealed bottles will be placed on a rotary shaker (150-200 rpm) and incubated in the dark at 20 – 25 °C (± 1°C).

4.5.3 Sampling

Following acidification of the medium (4.5.4), a sample of 1ml will be withdrawn from the headspace and injected into the gas chromatograph (GC) for the determination of CO_2 concentration. The sampling schedule will depend on the lag period and rate of biodegradation of the test substances. Three bottles will be sacrificed for analysis on each day of sampling, which will be approximately once every 5 days. The test should normally run for 28 days but might be prolonged if degradation has begun by day 28. On the last day of sampling, 5 bottles will be sacrificed. Bottles representing the inhibition and abiotic controls will be sampled only on day 1 and 28 (or last day).

4.5.4 Inorganic carbon (IC) analysis

CO_2 production in the bottles is determined by measuring the increase in the concentration of IC during incubation. 1 ml of concentrated H_3PO_4 (≥ 85%) will be injected through the septum of each bottle sampled in order to lower the pH of the medium to less than 3. Following the acid addition, the bottles will be shaken for at least an hour at the test temperature in order to achieve equilibrium between the liquid and gaseous phases. Then, 1 ml aliquots of the headspace will be withdrawn using a gas tight syringe and injected in a gas chromatograph (GC). The measured CO_2 concentrations will be recorded as mgC/L.

Standards: Standards for GC analysis will be prepared as follows: Na_2CO_3 solutions of 0, 5, 10 15 mgC/L will be prepared in CO_2 free water and dispensed in serum bottles in a liquid to gas ratio identical to the one used for the test. The solutions will be acidified with 1 ml H_3PO_4 and equilibrated for at least an hour. Aliquots from the headspace will be injected to the GC and the values obtained will be used as calibration standards for the equivalent carbon concentrations.

5. DATA AND REPORTING

5.1 Calculation of results

Assuming 100% mineralization of the test substance to CO_2, the maximum IC production (ThIC) in excess of blank production equals the amount of total organic carbon (TOC) contributed by the test substance added to each bottle. The total mass of inorganic carbon (TIC) in each bottle is the summation of the mass in the liquid and the mass in the headspace. The standards were prepared from defined IC solutions that were acidified and treated like the test samples. Therefore, each standard represents a specific carbon concentration in solution after equilibration with gaseous phase. Consequently, the TIC mass in the reaction mixtures equals the carbon concentration value obtained by the GC analysis multiplied by the liquid volume.

The percentage of biodegradation (%D) is given by the following equation:

$$\%D = (TIC_t - TIC_b) / TOC \times 100$$

where:

TIC_t = mg TIC in test bottle at time t

TIC_b = mean mg TIC in blank bottles at time t

TOC = mg TOC added initially to the test vessel

5.2 Expression of results

A biodegradation curve will be drawn by plotting percentage biodegradation D against time of incubation. If possible, the lag phase, the biodegradation phase, the 10-d window and plateau phase will be indicated. If comparable results will be obtained from parallel test vessels (<20% difference), a mean curve will be plotted. The value of maximal degradation and the value at the end of the 10-d window will be reported.

5.3 Validity of results

A test is considered valid if:

(a) The mean percentage degradation in the vessel containing the reference substance 1- octanol (B) is >60% by the 14th day of incubation ; and

(b) The mean amount of TIC present in the blank controls at the end of the test is <15% of the organic carbon added initially as the test substance.

5.4 Interpretation of results

(a) Biodegradation >60% ThIC within the 10-d window in this test demonstrates that the set substance is readily biodegradable under aerobic conditions.

(b) Biodegradation of <25% of reference substance in the inhibition controls (E and F) at the end of the test and insufficient degradation of test substance can indicate that the substance is inhibitory. In this case the test could be repeated using a lower substance test concentration.

(c) If test substance doesn't reach 60% ThIC biodegradation (20%-60%) and was shown not to be inhibitory, the test can be repeated with increased concentration of inoculum (up to 30 mg/L activated sludge) or inocula from other sources.

6. QUALITY ASSURANCE

Permanent records of all data generated during the course of this study, the protocol, protocol revisions/changes, the final report and correspondence relevant to this study between IMI and the sponsor will be archived at the IMI Wastewater Laboratory. In addition, all data generated and detailed experimental procedures will be recorded in a laboratory notebook and confirmed by signatures of both the laboratory technician and the study director on each page.

7. REFERENCES

1. Organization for Economic Cooperation and Development (OECD) (October 2003): Proposal for a new guideline 310 for Testing of Chemicals: Ready biodegradability – CO_2 in sealed vessels (Headspace test).
2. US EPA (January 1998): Fate, Transport and Transformation Test Guidelines: OPPTS 825.3120 Sealed-Vessels CO_2 Production Test.
3. Struijs. J., Stoltenkamp. J. (1990); Headspace determination of evolved carbon dioxide in a biodegradability screening test. Ecotoxicology and Environmental Safety 19, 204-211.

8. APPENDICES

8.1 Mineral medium

The following stock solutions (a), (b), (c), (d) will be prepared in distilled water. If a precipitate forms in a stock solution, it will be replaced with a freshly made solution.

Stock solution (a)

Compound	Stock concentration (g/L)
KH_2PO_4	8.5
K_2HPO_4	21.75
$Na_2HPO_4.2H_2O$	33.4
NH_4Cl	0.5
Dissolve in water and make up to 1 liter. The pH of the solution should be 7.4	

Stock solution (b)

Compound	Stock concentration (g/L)
$CaCl_2.2H_2O$	36.4
Dissolve in water and make up to 1 liter.	

Stock solution (c)

Compound	Stock concentration (g/L)
$MgSO_4.7H_2O$	22.5
Dissolve in water and make up to 1 liter.	

Stock solution (d)

Compound	Stock concentration (g/L)
$FeCl_3$	0.15
Dissolve in water and make up to 1 liter. One drop of concentrated HCl is added per liter to prevent precipitation of the solution	

The mineral medium will be prepared by mixing the stock solutions as follows:

1. Mix 10 ml of stock solution (a) with 800 ml CO_2 free water
2. Add 1 ml of each of the solutions (b), (c) and (d) and make up to 1 liter with CO_2 free water.

- Attachment 15 -

REPORT

Study Title

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH FR-1435X (FLOW-THROUGH)

Author

Ir. L.M. Bouwman

Study completion date

31 August 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419816
NOTOX Substance 146232/A

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

Tap water analyses were not performed under GLP and therefore excluded from this GLP statement.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: 31 August 2005

Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology

Date: 31/08/2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	protocol	14-Oct-04	14-Oct-04	14-Oct-04
	Amendment 1 of protocol	12-Jan-05	12-Jan-05	12-Jan-05
	Amendment 2 of protocol	02-Feb-05	02-Feb-05	02-Feb-05
	Amendment 3 of protocol	04-Feb-05	04-Feb-05	04-Feb-05
	Amendment 4 of protocol	11-Feb-05	11-Feb-05	11-Feb-05
	Amendment 5 of protocol	08-Apr-05	08-Apr-05	08-Apr-05
	Report	11-Apr-05	12-Apr-05	12-Apr-05
	Amendment 6 of protocol	26-Aug-05	26-Aug-05	26-Aug-05
Process	**Ecotoxicology & Biodegradation** Test substance handling Exposure Observations/Measurements	17-Jan-05	21-Jan-05	21-Jan-05
	Analytical and physical chemistry Test substance handling Observations/Measurements	10-Jan-05	14-Jan-05	20-Jan-05

Head of Quality Assurance
C.J.Mitchell B.Sc.

Date: 11/9/05

4. SUMMARY

Acute Toxicity Study in *Daphnia magna* with FR-1435X.

The study procedures described in this report were based on the ISO International Standard 6341, 1996. In addition, the procedures were designed to meet the test methods and validity criteria of the EEC directive 92/69, Part C.2, 1992, the OECD guideline No. 202 Part I, 1984 and the OECD series on testing and assessment number 23, 2000.

The batch of FR-1435X tested was a brown liquid with a purity of 97.2%. The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor). Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone.

The project started with a static range-finding test. Ten daphnids per concentration (in duplicate, 5 per vessel) were exposed to nominal FR-1435X concentrations of 0.05, 0.50 and 5.0 mg/l and to a solvent-control. After 48 hours of exposure 40 and 100% of the organisms exposed to nominal concentrations of 0.50 and 5.0 mg/l, respectively, were immobilised. No organisms were immobilised at the lower nominal test concentration of 0.05 mg/l. The expected EC_{50} was between nominal 0.50 and 5.0 mg/l. The analytical results obtained during the simultaneously performed static range-finding test with carp showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.40 and 3.7 mg/l, respectively. The lowest and the highest actual test concentrations decreased by more than 20% over the first 24 hours of exposure, while the 0.50 mg/l concentration remained stable. During the last 72 hours all concentrations decreased (NOTOX Project 419805). Hence, as 2 out of 3 concentrations decreased by more than 20% during the first 24-hour test period it was decided to continue testing applying a continuous renewal system for the final test.

The project was continued with a final test in a flow-through system. Target concentrations were 0.05, 0.10, 0.20, 0.50 and 1.1 mg/l. Twenty daphnids per concentration (in quadruplicate, 5 per mesh) were exposed per target concentration and a solvent-control for a maximum of 48 hours. The stock solutions in acetone were prepared daily and were dosed via a computer controlled system consisting of 6 dispensers (Gilson). Via this system the dosed volume entered a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter with a flow rate of ca. 12 l/h. In the mixing flask the dosed volume and the tap water were mixed under continuous stirring. Subsequently, the test solution coming from the mixing flask entered the vessel containing the daphnids. The dosing system was started two days before introduction of the organisms and the whole system was checked daily. Samples for analytical confirmation of actual test concentrations were taken 24 hours prior to the exposure period and at the start and the end of the 48-hour test period.

Analyses of the samples taken during the final test showed that the actual concentrations were generally in agreement with the target concentrations during the test period and stable within 20% of the start concentrations. Based on these results, the EC_{50} and the NOEC were based on mean exposure concentrations: 0.05, 0.08, 0.18, 0.33 and 0.91 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

FR-1435X did not induce acute immobilisation of *Daphnia magna* at a mean exposure concentration of 0.33 mg/l after 48 hours of exposure (NOEC).

The 48h-EC_{50} was 0.64 mg/l based on mean exposure concentrations (95% confidence interval between 0.57 and 0.78 mg/l). The 48h-EC_{50} was above the water solubility of FR-1435X.

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

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Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	
Water	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

The sponsor is responsible for all test substance data unless determined by NOTOX.

4.1.2. Reference substance

Results of a recently performed reference test with potassium dichromate will be included in the report. Reference tests will be performed once every three months.

4.2. Test system

Species	*Daphnia magna* (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.
Reason for selection	This system has been selected as an internationally accepted invertebrate species.
Validity of batch	Daphnids will originate from a healthy stock, 2nd to 5th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there should be no delay in the production of the first brood.

Characteristics	For the test selection of young daphnia with an age of < 24 hours, from parental daphnids of more than two weeks old.

4.3. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing about 250 of them into 5 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154, 25-33).

Composition of medium M7:

ISO medium: the following chemicals (analytical grade) are dissolved in tap water purified by reverse osmosis (milli-RO, Millipore Corp., Bedford, Mass., USA):

Macro salts:	$CaCl_2.2H_2O$	293.8	mg/l
	$MgSO_4.7H_2O$	123.3	mg/l
	$NaHCO_3$	64.8	mg/l
	KCl	5.8	mg/l

Medium M7: trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations:

Trace elements:	B	0.125	mg/l
	Fe	0.05	mg/l
	Mn	0.025	mg/l
	Li, Rb and Sr	0.0125	mg/l
	Mo	0.0063	mg/l
	Br	0.0025	mg/l
	Cu	0.0016	mg/l
	Zn	0.0063	mg/l
	Co and I	0.0025	mg/l
	Se	0.0010	mg/l
	V	0.0003	mg/l
	$Na_2EDTA.2H_2O$	2.5	mg/l
Macro nutrients:	$Na_2SiO_3.9H_2O$	10.0	mg/l
	$NaNO_3$	0.27	mg/l
	KH_2PO_4	0.14	mg/l
	K_2HPO_4	0.18	mg/l
Vitamins:	Thiamine	75.0	µg/l
	B_{12}	1.0	µg/l
	Biotin	0.75	µg/l

The hardness: 250 mg/l expressed as $CaCO_3$ and the pH: 8.0 ± 0.2 after aeration.

4.4. Preparation of stock and test solutions

The exact procedure for preparation of test solutions will be based on a pretest, of which the result will be kept in the raw data.

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable, and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance on the water surface or extensive precipitation, flocculation, aggregation or deposition of the undissolved fraction of the test substance. The method of preparation of the test solutions will be based on data of the test substance supplied by the sponsor and/or on the results of a preliminary test (or specific tests with the test substance performed by NOTOX when the sponsor requests these).

The method of preparation will be based on the principles laid down in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and referred to in the OECD Guidance Document On The Use Of The Harmonised System For The Classification Of Chemicals Which Are Hazardous For The Aquatic Environment, section 3.5: "Difficult to test substances".

The tests will be carried out without adjustment of the pH, except if pH values of the stocks are outside the range of 3-10 and are expected to induce similar extreme pH values in the test solutions.

4.5. Range-finding test

A range-finding test will be performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions will be similar to those applied in the final test with the following exceptions:
Ten daphnia per concentration (5 per vessel) will be exposed to a range of 0.1 to 100 mg/l increasing by a factor of 10. If applicable a range will be tested up to and including the maximum solubility if this is below 100 mg/l. Dissolved oxygen concentrations and pH will at least be measured in the control and the highest test concentration. No sampling for determination of actual test concentrations will be performed.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

If no toxicity is expected, based on the characteristics of the test substance or other specific information, a limit test or alternatively a limit test combined with a range-finding test will be performed.

4.5.1. Limit test

The limit test will consist of a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Test procedure and conditions will be similar to those applied in the final test. Samples for determination of actual exposure concentrations will be taken from the control and the test concentration at the start and the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium different methods of preparation may be combined in order to reach the expected water solubility.

4.5.2. Combined limit/range-finding test

In a combined limit/range-finding test, twenty Daphnia per concentration will be exposed to a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Ten daphnia per concentration will be exposed to 0.1, 1.0 and 10 mg/l, or if applicable dilutions containing 0.1, 1.0 and 10% of the saturated solution. Dissolved oxygen concentrations and pH will at least be measured in the control and the highest test concentration. Samples for determination of actual exposure concentrations will be taken from the control and the highest test concentration at the start and the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

4.6. Final test

4.6.1. Test concentrations

Number	At least 5 concentrations in a geometric series with a factor ≤ 2.2, except when the EC_{50} is expected to be greater than the maximum concentration to be tested. In that case a limit test can be performed.
Range	The concentration range to be tested has to cover at least one concentration causing no immobility, one concentration causing 100% immobilisation with a maximum concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower, and a sufficient number of different concentrations expected to induce immobility rates between 10% and 90%.
Controls	Test medium without test substance but treated in the same way as the test substance solutions.

4.6.2. Test procedure and conditions

Test duration	48 hours
Test type	Static
Test vessels	100 ml, all-glass
Medium	ISO
Number of daphnia	20 per concentration
Loading	5 per vessel (with ca. 80 ml of test solution)
Light	16 hours photoperiod daily; should the test substance be light sensitive, the test will be performed in the dark.
Temperature	18-22°C, constant within 2°C.

Oxygen concentration	> 7 mg/l at the start of the test, ≥ 5 mg/l at the end of the test.
pH	Between 6.0 and 8.5. Should not vary by more than 1 unit at the end of the test in any test solution.
Feeding	No feeding
Aeration	No aeration of the test solutions.
Introduction of daphnia	Daphnia are introduced into the test medium as soon as possible after preparation of the test solutions.

4.6.3. Sampling for analysis of test concentrations

Frequency	At the start (t=0 h) and the end of the test (t=48 h).
Concentrations (standard[1])	Samples for analysis will be taken from three concentrations, i.e. the lowest, a middle and the highest, and the control. At the start of the test care will be taken not to include any floating layer, test substance film or undissolved material in separate vessels. At the end of the test samples will be taken from the approximate centre of the pooled solutions (provided that solutions are still homogeneous) of the vessels at each concentration.
Number of samples	Sampling will consist of singular samples per treatment. Should the analytical validation require duplicate or multiple samples per treatment, this will be followed without prior notification.
Volume	Standardly, volumes of 700 µl will be taken, but depending on the limit of detection of the analytical method used in relation to the test concentrations the volume may differ.
Storage	If stability of test concentrations under deep-freeze conditions is ensured, the samples will be stored in a deep-freezer until analysis. Optionally, samples can be stored under different conditions (e.g. room temp. or in refrigerator) if stability under these conditions is ensured.
Extra samples	In case singular samples are taken, which are known to be stable under the storage conditions, extra samples will be taken from all concentrations and stored for possible analysis until delivery of the final report with a maximum of three months.

[1] The standard frequency of sampling is only applicable provided that test solutions are diluted using **one** stock and test concentrations should be **above** 1 mg/l. Sampling will include **all** test solutions if the previous mentioned conditions are not met or if the Study Director decides this is essential for other reasons. Alternatively, sampling may be limited to those test solutions that are biologically relevant.

Analyses	When used for analysis the entire volume of each sample will be taken for further dilution or pre-treatment. The analytical method used will be based on the results of a separate project for the development and validation of the analytical method. If study specific adjustments of the analytical method or sample pre-treatment procedures are necessary, these will be developed and tested before the performance of the final test. Detailed specification of these additional analytical procedures will be put down in a protocol amendment (see also 'Additional procedures').

4.6.4. Measurements and recording

Immobility (including mortality)	At 24 hours and at 48 hours.
pH	At the beginning and at the end of the test, for all concentrations and the control.
Dissolved oxygen	At the beginning and at the end of the test, for all concentrations and the control. In addition after 24 hours, immediately after counting the immobilized daphnids oxygen levels will be measured in the test container with the solution of lowest concentration at which all the *Daphnia magna* have been immobilized.
Temperature of medium	Continuously in a temperature control vessel, beginning at the start of the test.

4.7. Interpretation

4.7.1. Acceptability of the test

1. In the control, not more than 10% of the daphnia should have been immobilised or trapped at the surface of the water.
2. The oxygen concentration will be ≥ 5 mg/l at the end of the test. Other test conditions (pH and temperature) will be maintained within the limits prescribed by this protocol.
3. The 24h-EC_{50} (based on the initial concentration) of potassium dichromate is within the range 0.6 mg/l to 1.7 mg/l.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

4.7.2. Additional procedures

Additional or alternative procedures will be required for the testing of:
1. Volatile substances;
2. Very toxic or low soluble substances (test concentrations < 1 mg/l);
3. Hydrolytically unstable substances;
4. pH affecting substances;
5. Substances that are not stable under deep-freeze conditions.

These additional procedures may require the amending of:
1. Preparation of test solutions;
2. Additional testing for determination of stability of exposure concentrations;
3. Procedures for maintenance of exposure concentrations (semi-static or flow-through system);
4. The frequency of sampling and analysis for the determination of actual test concentrations;
5. Extension of the analytical program with respect to sample treatment and the sensitivity of the analytical method;
6. If applicable, additional testing with pH adjustment.

The additional procedures are no part of the standard test procedures and will be applied only after emission of an authorised protocol amendment. In such a case the amended procedures will be effective only after NOTOX has received any kind of authorisation from the study monitor.

4.7.3. Data handling

Defining exposure concentrations
1. The results will be based on the nominal or initial (if not in agreement with nominal) test substance concentrations if the analytical program has confirmed that the measured test substance concentrations remained within 20% of the nominal or initial concentrations.
2. If the deviation of the exposure concentrations of the test substance is greater than ± 20% of the nominal or initial concentrations, the results will be expressed in terms of average exposure concentrations. Where measured data are available for the start and end of the test, these concentrations are geometric means calculated from the concentrations measured at the start and end of the test. Where at the end of the test measured concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit.

Calculation of EC_{50}
If possible, an EC_{50}-value will be calculated at 24 and 48 hours of exposure from the probits of the percentages of affected daphnia and the logarithms of the corresponding nominal concentrations using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition) or using the Logit-model (Cox, D.R., 1977: Analysis of binary data, Methuen & Co. Ltd.). Optional: data will be analysed using the DEBTOX-model for generation of NEC and EC_x-values as described in 'The Analysis of Aquatic Toxicity Data' by S.A.L.M. Kooijman and J.J.M. Bedaux, 1996.

Should there be no concentration between the highest concentration (A) at which 0% immobility has occurred and the lowest concentration (B) at which 100% immobility has occurred, the EC_{50} will be calculated as $(AB)^{1/2}$, where A and B are limits of the 95% confidence interval.

No EC_{50} can be calculated if the test substance proves to be non-toxic (EC_{50} > maximum concentration).

Optionally, other statistical analyses may be performed if appropriate.

5. DISTRIBUTION

Original: Study Director
1 Copy: Technical Coordinator
1 Copy : Analytical Chemistry
1 Copy: QAU/Management
1 Copy: Sponsor

FR-1435X

NOTOX Project 419816

APPENDIX IV CERTIFICATE OF ANALYSIS

IMI (TAMI) Institute for Research & Development Ltd
Flame Retardants Department
P.O.Box 10140, Haifa Bay 26111, Israel Tel.+972-4-8469553
Fax:+972-4-846-9320 E-mail:zilbermanj@tami-imi.co.il

תמי (אימי) מכון למחקר ולפתוח בעימ
מחלקת מעכבי בעירה
ת.ד. 10140, מפרץ חיפה 26111 טל. 04-8469553
E-mail: zilbermanj@tami-imi.co.il 04-8469320 :פקס

To: Lilach Yakobovich - DSBG

From: Y. Zilberman - IMI

Date: 14 December 2003

Following are some of the characteristics of the products.

CONFIDENTIAL

APPENDIX V

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH FR-1435X (FLOW-THROUGH); DETERMINATION OF THE CONCENTRATIONS

Author

Dr. Ir. C. Brands

Study completion date

August 31, 2005

Laboratory Project Identification

NOTOX Project 419816
NOTOX Substance 146232/A

1. CONTENTS

TABLES

FIGURES

2. REPORT APPROVAL

PRINCIPAL SCIENTIST: Dr. Ir. C. Brands
(Analytical Chemistry)

C Brands

Date: August 31, 2005

3. INTRODUCTION

3.1. Preface

Study plan (analytical study)	Start	: 7 March 2005
	Completion	: 11 March 2005

3.2. Aim of the study

The purpose of the analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Methanol	HPLC-grade, Labscan, Dublin, Ireland
Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore Corp, Bedford, MA, USA
Tap water	see main report

4.2. Samples

The samples (10 ml) were diluted in a 1:1 (v:v) ratio with methanol and thereafter ultrasonicated for 5 minutes.

4.3. Analysis

4.3.1. Analytical method

Quantitative analysis was based on the sum of both peaks in the HPLC chromatogram of FR-1435X (See NOTOX Project 419838: "Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium").

Analytical conditions

Column	
Stationary phase	Zorbax RX-C18
Dimensions	250 x 4.6 mm; dp = 5 µm
Brand	Agilent, Palo Alto, CA, USA
Mobile phase	90/10 (v/v) methanol/Milli-Q water
Flow	1.2 ml/min
Detection	Spectrophotometric (wavelength of 225 nm)
Injection volume	100 µl

4.3.2. Calibration

On the first day of analysis, calibration solutions in 50/50 (v/v) methanol/tap water were made up from two standard solutions of FR-1435X in methanol. Standard solutions were ultrasonicated for 15 minutes in order to dissolve the test substance.

4.3.3. Injections

Calibration solutions were injected in duplicate. Procedural recovery samples and test samples were analysed by single injection.

4.3.4. Data acquisition and data processing

Data acquisition and data processing was performed using Empower software (Waters, Milford, MA, USA) version 5.00.

4.3.5. Procedural recovery samples

Standard solutions in methanol and dilutions of these solutions with methanol were used as spiking solutions. Standard solutions were ultrasonicated for 15 minutes in order to dissolve the test substance.

On each day of analysis, blank test medium (10 ml) was spiked at concentration levels of approximately 0.025 mg/l, 0.5 mg/l and 1.1 mg/l [1] using spiking solutions with concentrations between 3.31 and 72.7 mg/l. At all concentration levels, samples were prepared and treated as described in paragraph 4.2 'Samples' (i.e. diluted in a 1:1 (v:v) ratio with methanol) and analysed. The recovery was calculated for each sample.

4.4. Interpretation

4.4.1. Calculations

All calculations were performed using non-rounded values. Rounded values are reported in the tables. Therefore, some differences might be observed when calculating the parameters as mentioned in the tables.

4.4.2. Formulas

General

Mean:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

where
x_i = measured value
n = number of measurements

Standard deviation

$$s_{n-1} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$$

Coefficient of variation

$$\frac{s_{n-1}}{\bar{x}} * 100\%$$

[1] Concentrations were not corrected for the spiking volume.

Calibration

Response: Peak area of test substance Peak 1 and Peak 2 [units]

Calibration curve: The response was correlated with the test substance concentration, using linear regression analysis (least squares method; weighting factor ($1/concentration^2$)).

$$R = a * C + b$$

R = response calibration solution [units]
C = concentration of test substance in calibration solution [mg/l]
a = slope [units*l/mg]
b = intercept [units]

Samples

Concentration of FR-1435X analysed in the samples:

$$C = \frac{(R - b) * d}{a} \quad [mg/l]$$

R = response sample [units]
d = dilution factor
a = slope [units*l/mg]
b = intercept [units]

Recovery of procedural recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100\ [\%]$$

Relative to target concentration:

$$\frac{\text{Concentration analysed}}{\text{Concentration target}} * 100\ [\%]$$

Relative to initial concentration:

$$\frac{\text{Concentration analysed at t = x hours}}{\text{Mean concentration analysed at t = 0 h}} * 100\ [\%]$$

5. RESULTS

5.1. Calibration curves

A calibration curve was constructed using four concentration levels. At each concentration level, two responses were used. The coefficient of correlation was in excess of 0.99.

Figure 1 shows chromatograms of a calibration solution and blank 50/50 (v/v) methanol/tap water.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are summarized in Table 1. Figure 2 shows chromatograms of procedural recovery samples.

Mean recoveries were between 102% and 110%, which indicated that analysis was performed properly.

5.2.2. Test samples

The results for the test samples are summarized in Table 2. Figures 3 – 5 show chromatograms of solvent-control samples, target 0.05 mg/l samples and target 1.1 mg/l samples.

TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration nominal [mg/l]	Concentration analysed [mg/l]	Recovery [%]	Mean recovery (Coefficient of variation) [%]
08-03-05	08-03-05	0.0251	0.0270	107	103
		0.0251	0.0245	98	
		0.502	0.543	108	109
		0.502	0.547	109	
		1.10	1.19	109	108
		1.10	1.19	108	(0.29)
		1.10	1.19	108	
09-03-05	09-03-05	0.0251	0.0251	100	102
		0.0251	0.0262	105	
		0.502	0.554	110	110
		0.502	0.550	110	
		1.10	1.20	109	109
		1.10	1.20	109	(0.36)
		1.10	1.20	110	
11-03-05	11-03-05	0.0228	0.0255	112	107
		0.0228	0.0235	103	
		0.456	0.496	109	110
		0.456	0.504	111	
		1.00	1.07	107	108
		1.00	1.09	109	(0.84)
		1.00	1.08	108	

Table 2 Concentrations of FR-1435X in test medium

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Target [mg/l]	Analysed [mg/l]	Relative to target [%]	Relative to initial [%]
-24	08-03-05	08-03-05	0 [1]	n.d.	n.a.	
			0 [1]	n.d.	n.a.	
			0.05	0.0552	110	
			0.05	0.0555	111	
			0.10	0.0829	83	
			0.10	0.0966	97	
			0.20	0.181	90	
			0.20	0.185	93	
			0.50	0.320	64	
			0.50	0.331	66	
			1.1	0.868	79	
			1.1	0.874	79	
			1.1	0.871	79	
0	09-03-05	09-03-05	0 [1]	n.d.	n.a.	
			0 [1]	n.d.	n.a.	
			0.05	0.0485	97	
			0.05	0.0515	103	
			0.10	0.0832	83	
			0.10	0.0837	84	
			0.20	0.175	88	
			0.20	0.177	89	
			0.50	0.332	66	
			0.50	0.332	66	
			1.1	0.894	81	
			1.1	0.906	82	
			1.1	0.894	81	
48	11-03-05	11-03-05	0 [1]	n.d.	n.a.	
			0 [1]	n.d.	n.a.	
			0.05	0.0482	96	96
			0.05	0.0553	111	111
			0.10	0.0712	71	85
			0.10	0.0713	71	85
			0.20	0.178	89	101
			0.20	0.177	88	100
			0.50	0.333	67	100
			0.50	0.341	68	103
			1.1	0.917	83	102
			1.1	0.917	83	102
			1.1	0.923	84	103

[1] Solvent control.

n.d. Not detected.

n.a. Not applicable.

FIGURES

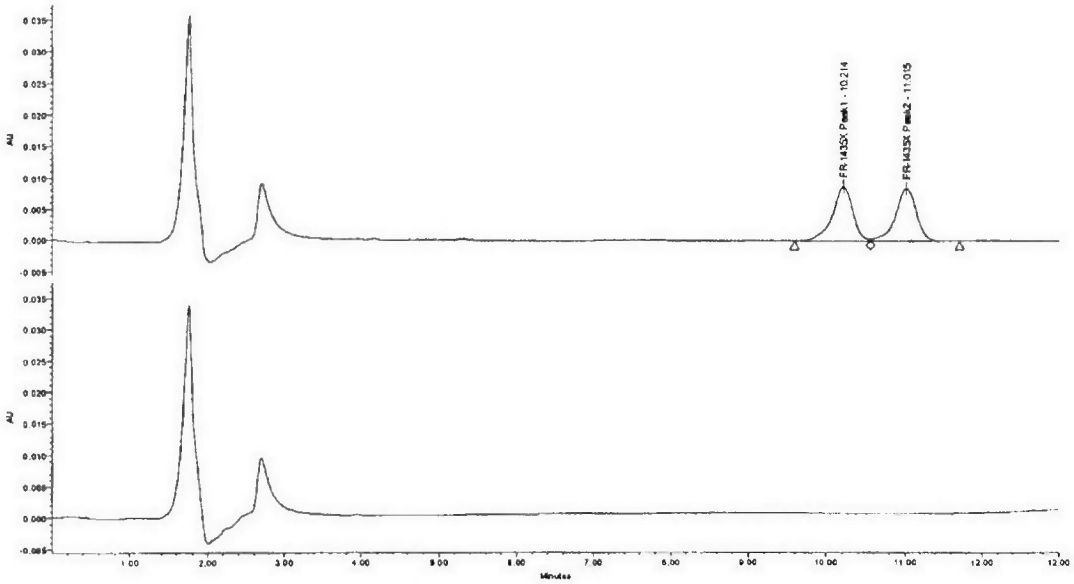


Figure 1 HPLC chromatograms of a 0.800 mg/l calibration solution (top) and blank 50/50 (v/v) methanol/tap water (bottom) [res.id. 3284 and 3302].

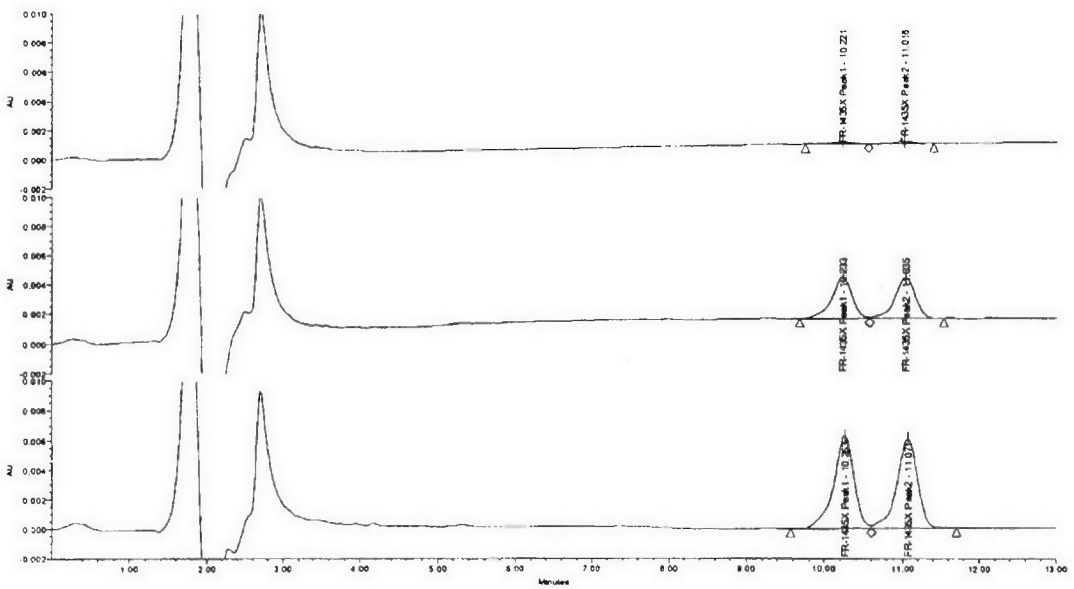


Figure 2 HPLC chromatograms of procedural recovery samples at concentrations of 0.0251 mg/l [top; res.id. 3319], 0.502 mg/l [middle; res.id. 3321] and 1.10 mg/l [bottom; res.id. 3323].

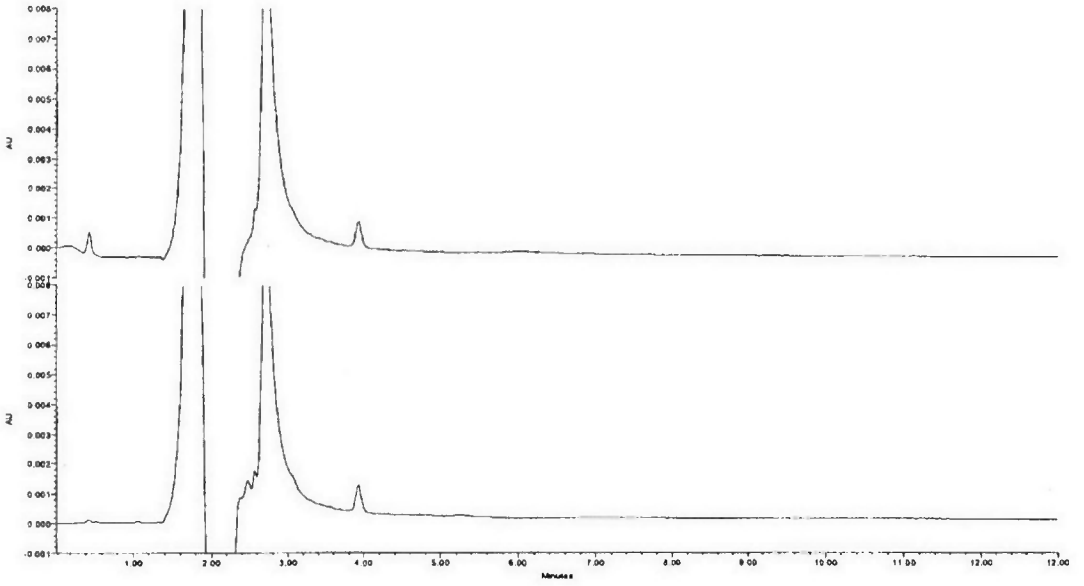


Figure 3 HPLC chromatograms of solvent-control samples taken at t=0 hours [top; res.id. 3350] and at t=48 hours [bottom; res.id. 3575].

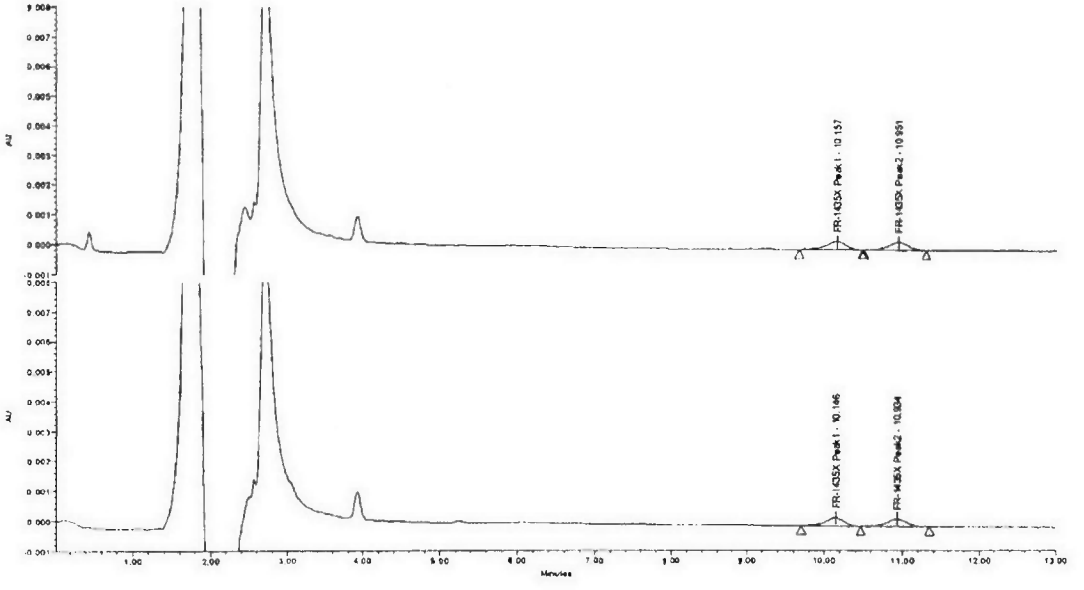


Figure 4 HPLC chromatograms of target 0.05 mg/l samples taken at t=0 hours [top; res.id. 3360] and at t=48 hours [bottom; res.id. 3577].

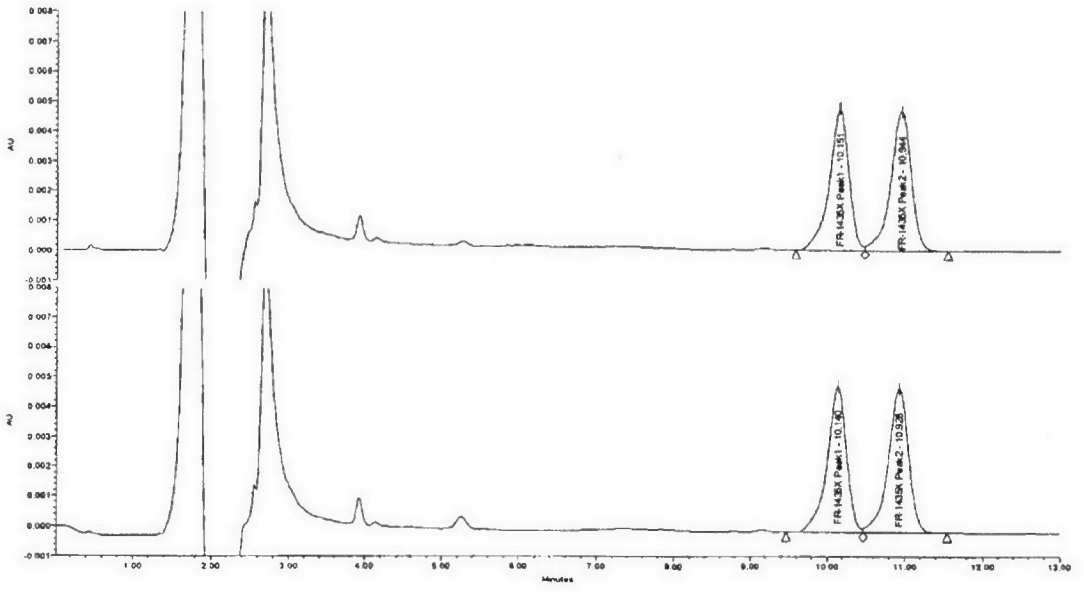


Figure 5 **HPLC chromatograms of target 1.1 mg/l samples taken at t=0 hours [top; res.id. 3373] and at t=48 hours [bottom; res.id. 3650].**

APPENDIX VI COPY OF THE PROTOCOL + AMENDMENT

PROTOCOL

Study Title

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA* WITH FR-1435X (STATIC)

Author

Ir. L.M. Bouwman

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419816
NOTOX Substance 146232/A

1. CONTENTS

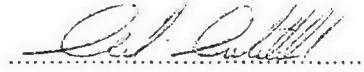
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Ir. L.M. Bouwman

.....

date: 13 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



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date: 14-10-04

SPONSOR: Dead Sea Bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

.....

date: 19.10.04

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	Ing. B. van Wees
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start week beginning : 08 November 2004 (week 46) Completed week beginning : 13 December 2004 (week 51) Proposed Reporting date : 06 February 2005

3.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate acute toxic effects on the mobility of *Daphnia magna* during an exposure period of 48 hours and, if possible, to determine the EC_{50} at 24 and 48 hours of exposure.

3.3. Guidelines

The study procedures described in this protocol are based on the ISO International Standard 6341: "Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test, Third edition, 1996-04-01.

In addition, the procedures are designed to meet the test methods and validity criteria prescribed by the following guidelines and guidance documents:

- European Community (EC), Commission directive 2004/73/EC, Part C: Methods for the determination of ecotoxicity, EC Publication No. L152, April 2004, C.2. "Acute Toxicity for Daphnia".
- Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202 Part I: "*Daphnia sp.*, Acute Immobilisation Test", Adopted April 4, 1984.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

3.7. Definitions

Immobile are those animals not able to swim within 15 seconds after gentle agitation of the test vessel.

The EC_{50} is the concentration of test substance estimated to immobilise 50% of the daphnia after a defined period of exposure.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which no effect (i.e. immobilisation) is recorded.

If appropriate, additional definitions may be included in the report (e.g. definitions referring to poorly soluble substances).

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	Ing. B. van Wees
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start : 22 November 2004 Completion : 11 March 2005

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate acute toxic effects on the mobility of *Daphnia magna* during an exposure period of 48 hours and, if possible, to determine the EC_{50} at 24 and 48 hours of exposure.

5.3. Guidelines

The study procedures described in this report were based on the ISO International Standard 6341: "Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus - Acute toxicity test, Third edition, 1996-04-01.

In addition, the procedures were designed to meet the test methods and validity of the following guidelines and guidance documents:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.2. "Acute Toxicity for *Daphnia*".
- Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202 Part I: "*Daphnia sp.*, Acute Immobilisation Test", Adopted April 4, 1984.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Immobile are those animals not able to swim within 15 seconds after gentle agitation of the test vessel.

The **EC_{50}** is the concentration of test substance estimated to immobilise 50% of the daphnia after a defined period of exposure.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which no effect (i.e. immobilisation) is recorded.

A **Flow-through test** is a test in which the water containing the test substance is renewed continuously.

6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Test substance

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000 kg/m^3
Stability in water	Not indicated

6.1.2. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (Appendix II).

6.1.3. Preliminary data

The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor). FR-1435X concentrations in the range of 0.0254 and 5.08 mg/l in ISO-medium showed to be stable during freezing and thawing of samples (NOTOX Project 419838).

6.2. Test system

Species	*Daphnia magna* (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.
Reason for selection	This system has been selected as an internationally accepted invertebrate species.
Validity of batch	Daphnids originated from a healthy stock, 2nd to 5th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood.
Characteristics	For the test selection of young daphnia with an age of < 24 hours, from parental daphnids of more than two weeks old.

6.3. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing about 250 of them into 5 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154, 25-33).

Composition of medium M7:

ISO medium: the following chemicals (analytical grade) are dissolved in tap water purified by reverse osmosis (milli-RO, Millipore Corp., Bedford, Mass., USA):

Macro salts:	$CaCl_2.2H_2O$	293.8	mg/l
	$MgSO_4.7H_2O$	123.3	mg/l
	$NaHCO_3$	64.8	mg/l
	KCl	5.8	mg/l

Medium M7: trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations:

Trace elements:	B	0.125	mg/l
	Fe	0.05	mg/l
	Mn	0.025	mg/l
	Li, Rb and Sr	0.0125	mg/l
	Mo	0.0063	mg/l
	Br	0.0025	mg/l
	Cu	0.0016	mg/l
	Zn	0.0063	mg/l
	Co and I	0.0025	mg/l
	Se	0.0010	mg/l
	V	0.0003	mg/l
	$Na_2EDTA.2H_2O$	2.5	mg/l
Macro nutrients:	$Na_2SiO_3.9H_2O$	10.0	mg/l
	$NaNO_3$	0.27	mg/l
	KH_2PO_4	0.14	mg/l
	K_2HPO_4	0.18	mg/l
Vitamins:	Thiamine	75.0	µg/l
	B_{12}	1.0	µg/l
	Biotin	0.75	µg/l

The hardness: 250 mg/l expressed as $CaCO_3$ and the pH: 8.0 ± 0.2 after aeration.

6.4. Static range-finding test

Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone (99.8%, Labscan, Ireland) at concentrations a factor of 10,000 above the nominal concentrations in test medium. Subsequently, amounts of 0.5 ml were added to 5 l ISO-medium to obtain the final test concentrations of 0.05, 0.50 and 5.0 mg/l. After a magnetic stirring period of 18 minutes the resulting solutions were clear and colourless, except for the 5.0 mg/l concentration that was slightly hazy.

The test solutions originated from those used for the simultaneously performed acute fish toxicity test (NOTOX Project 419805).

A static range-finding test was performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those applied in the final test with the following exceptions: Ten daphnia per concentration (in duplicate, 5 per vessel) were exposed to 0.05, 0.50 and 5.0 mg/l and to a solvent-control. Test solutions were not renewed during the test period. Dissolved oxygen concentrations and pH were only measured in the solvent-control and the highest test concentration at the start and the end of the test. No sampling for determination of actual test concentrations was performed.

6.5. Flow-through final test

6.5.1. Test concentrations

FR-1435X	0.05, 0.10, 0.20, 0.50 and 1.1 mg/l
Controls	Test medium without test substance but containing acetone as used in the treatment of the stock solutions (solvent-control).

6.5.2. Test procedure and conditions

Test duration	48 hours
Test type	Flow-through, continuous renewal of test media.
Test vessels	10 litres, all-glass containing four stainless steel meshes
Medium	Tap water (see Appendix III for data of analysis)
Number of daphnia	20 per concentration
Loading	5 per mesh
Light	16 hours photoperiod daily
Feeding	No feeding
Aeration	No aeration of the test solutions.
Introduction of daphnia	Two days after the start of the dosing to permit stabilisation of the system

6.5.3. Preparation of stock solutions

Due to the low water solubility of the test substance, stock solutions in acetone (99.8%, Labscan, Ireland) were prepared at concentrations a factor of 10,000 above the target concentrations in test medium. These stocks were used for dosing and were prepared daily.

6.5.4. Dosing system

The stock solutions in acetone were dosed via a computer controlled system consisting of 6 dispensers (Gilson). Via this system the dosed volume entered a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter with a flow rate of ca. 12 l/h. In the mixing flask the dosed volume and the tap water were mixed under continuous stirring. Subsequently, the test solution coming from the mixing flask entered the vessel containing the daphnids. The whole system was checked daily. The dosing program is described in Table 1 and a diagram of the system is presented in Figure 1 on the next page.

Table 1 Dosing program

Stock solution FR-1435X in acetone (g/l)	Dosing volumes (0.5 ml syringe)		Target concentrations FR-1435X (mg/l)
	Per cycle ml/5 min	Per hour ml/hour	
0.5	0.10	1.2	0.05
1.0	0.10	1.2	0.10
2.0	0.10	1.2	0.20
5.0	0.10	1.2	0.50
11	0.10	1.2	1.1

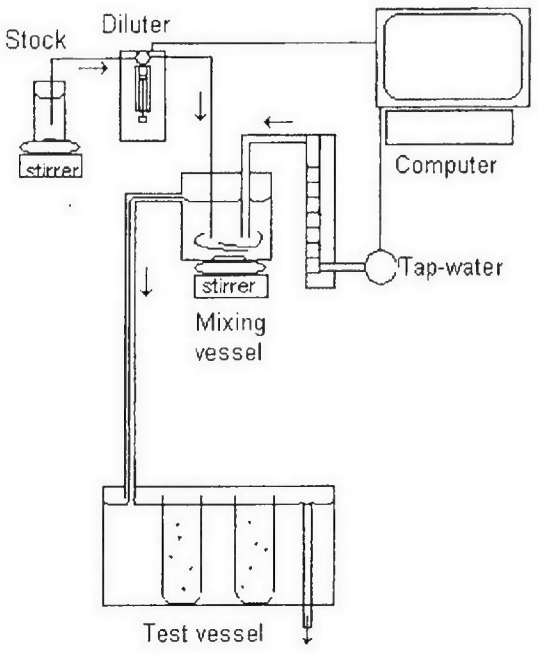


Figure 1 Flow-through system

6.5.5. Sampling for analysis of test concentrations

During the final test duplicate samples were taken from concentrations < 1.0 mg/l and triplicate samples were taken from concentrations ≥ 1.0 mg/l. These samples were used for analyses and were taken according to the schedule below. The method of analysis is described in the appended Analytical Report (Appendix V).

Sampling: Frequency	24 hours following the start of dosing, samples were taken from all target concentrations and the control to check the actual exposure concentration. At the start and the end of the exposure period samples were taken from all target concentrations and the control.
Volume	10 ml from the approximate centre of the test vessel
Storage	Not applicable, samples were analysed on the day of sampling.

6.5.6. Measurements and recordings

Immobility (including mortality)	At 24 hours and at 48 hours.
pH and dissolved oxygen	At the beginning, after 24 hours and at the end of the test, for all target concentrations and the control.
Temperature of medium	Continuously in a temperature control vessel, beginning at the start of the test.

6.6. Interpretation

6.6.1. Data handling

Defining exposure concentrations:

The results were based on the mean of the concentrations measured at the various sampling intervals during the exposure period.

Calculation of EC_{50}:

The EC_{50}-value was calculated at 48 hours of exposure from the probits of the percentages of affected daphnia and the logarithms of the corresponding test substance concentrations using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition).

6.6.2. Acceptability of the test

1. In the control, no daphnia became immobilised or trapped at the surface of the water.
2. The oxygen concentration was ≥ 5 mg/l at the end of the test. Other test conditions (pH and temperature) were maintained within the limits prescribed by the guidelines.
3. The 24h-EC_{50} (based on the initial concentration) of potassium dichromate was within the range 0.6 mg/l to 1.7 mg/l.

6.7. List of protocol deviations

1. For the final test glass vessels of 10 litre were used, containing 4 mesh containers of stainless steel, covered by a removable Perspex plate.
   Evaluation: By using larger test vessels, the total volume change of the test vessels per time unit is decreased.
2. During the first 24 hours of the flow through final test 3 daphnids exposed to the target concentration of 0.50 mg/l were lost in the system.
   Evaluation: 17 instead of 20 daphnids were used in the statistical evaluation of the results.
3. During the flow-through final test the hardness of tap water was 120 mg/l $CaCO_3$.
   Evaluation: This value was within the recommended range for hardness of dilution water (i.e. 80-250 mg/l $CaCO_3$) and therefore did not affect the daphnids.

The study integrity was not adversely affected by these deviations.

7. RESULTS

7.1. Static range-finding test

Table 2 shows the responses recorded during the range-finding test. After 48 hours of exposure 40 and 100% of the organisms exposed to nominal concentrations of 0.50 and 5.0 mg/l, respectively, were immobilised. No organisms were immobilised at the lower nominal test concentration of 0.05 mg/l. The expected EC_{50} was between nominal 0.50 and 5.0 mg/l. All test conditions were maintained within the limits prescribed by the protocol.

The analytical results obtained during the simultaneously performed static range-finding test with carp showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.40 and 3.7 mg/l, respectively. The lowest and the highest actual test concentrations decreased by more than 20% over the first 24 hours of exposure, while the 0.50 mg/l concentration remained stable. During the last 72 hours all concentrations decreased (NOTOX Project 419805). Hence, as 2 out of 3 concentrations decreased by more than 20% during the first 24-hour test period it was decided to continue testing applying a continuous renewal system for the final test.

Table 2 Incidence of immobility in the static range-finding test

Nominal conc. FR-1435X (mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Solvent-control	A	5	0	0	0	0
	B	5	0		0	
0.05	A	5	0	0	0	0
	B	5	0		0	
0.50	A	5	1	40	1	40
	B	5	3		3	
5.0	A	5	5	90	5	100
	B	5	4		5	

7.2. Flow-through final test

7.2.1. Measured concentrations

The results of analysis of the samples taken during the flow-through final test are described in Table 2 of the appended Analytical Report.

Analyses of the samples taken during the final test showed that the actual concentrations were generally in agreement with the target concentrations during the test period and stable within 20% of the start concentrations. Based on these results, the EC_{50} and the NOEC were based on mean exposure concentrations: 0.05, 0.08, 0.18, 0.33 and 0.91 mg/l.

7.2.2. Immobility

Table 3 shows the responses recorded during the flow-through final test. The responses recorded in this test allowed for reliable determination of an EC_{50}. Note that 5% immobility at 0.18 mg/l was considered insignificant as a maximum response of 10% is acceptable for the control and no immobility was observed at 0.33 mg/l.

Table 3 Acute immobilisation of daphnia after 24 and 48 hours in the flow-through final test

Mean conc. FR-1435X (mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Solvent-control	A	5	0	0	0	0
	B	5	0		0	
	C	5	0		0	
	D	5	0		0	
0.05	A	5	0	0	0	0
	B	5	0		0	
	C	5	0		0	
	D	5	0		0	
0.08	A	5	0 (1)	0	0	0
	B	5	0		0	
	C	5	0		0	
	D	5	0		0	
0.18	A	5	0	0	0	5
	B	5	0		0	
	C	5	0		1	
	D	5	0		0	
0.33	A	5	0	0	0	0
	B	5[1]	0		0	
	C	5	0		0	
	D	5	0		0	
0.91	A	5	0 (3)	20	4	85
	B	5	0		5	
	C	5	3 (5)		3	
	D	5	1 (3)		5	

[1] During the first 24 hours of exposure 3 daphnids were lost in the flow-through system
Between brackets: number of daphnids trapped at the surface. These organisms were reimmersed into the respective solutions before recording of immobility.

7.2.3. Determination of effect concentrations

Table 4 shows the effect parameters based on mean concentrations, see also Appendix I.

Table 4 Effect parameters

Parameter	Concentration FR-1435X (mg/l)	95%-confidence interval
NOEC	0.33	
24h-EC_{50}	> 0.91	
48h-EC_{50}	0.64	0.57 - 0.78

7.2.4. Experimental conditions

The results of measurement of pH and oxygen concentrations (mg/l) are presented in Table 5. These test conditions remained within the limits prescribed by the protocol (pH: 6.0-8.5, not varying by more than 1 unit; oxygen: >7 mg/l at the start, ≥5 mg/l at the end of the test).

The temperature of the test medium was 21.0°C at the start of the test. The temperature continuously measured in a temperature control vessel varied between 20.5 and 21.3°C during the test, and complied with the requirements as laid down in the protocol (18-22°C, constant within 2°C).

Table 5 pH and oxygen concentrations (mg/l) during the flow-through final test

Mean conc. FR-1435X (mg/l)	Start (t=0 h)		t=24h		End (t=48 h)	
	pH	O_2	pH	O_2	pH	O_2
Solvent-control	7.9	9.9	7.9	9.9	8.0	9.9
0.05	7.9	10.0	7.9	9.9	7.8	9.9
0.08	7.9	9.8	7.9	9.8	8.0	9.9
0.18	7.9	10.0	7.9	9.9	7.9	9.8
0.33	7.9	9.8	7.9	9.9	8.0	9.8
0.91	7.9	9.8	7.9	9.8	8.0	9.9

8. CONCLUSION

Under the conditions of the present study FR-1435X did not induce acute immobilisation of *Daphnia magna* at a mean exposure concentration of 0.33 mg/l after 48 hours of exposure (NOEC).

The 48h-EC_{50} was 0.64 mg/l based on mean exposure concentrations (95% confidence interval between 0.57 and 0.78 mg/l). The 48h-EC_{50} was above the water solubility of FR-1435X.

APPENDIX I EC-VALUES

Table 6 EC_{50} value at 48 hours and related parameters

$48h\text{-}EC_{50}$ = 642.4 µg/l
95 % fiducial limits: 568.9 - 777.5 µg/l

index of regression significance: g=0.05
chi-squared=4.31, with 6 degrees of freedom

regression line: log10(conc.)=2.52+(probit-3.03)/6.84

conc. µg/l	group size	response	corrected fraction	expected fraction	chi2
330	5	0	0.00	0.00	0.00
330	2	0	0.00	0.00	0.00
330	5	0	0.00	0.00	0.00
330	5	0	0.00	0.00	0.00
910	5	4	0.80	0.85	0.10
910	5	5	1.00	0.85	0.88
910	5	3	0.60	0.85	2.45
910	5	5	1.00	0.85	0.88
					4.31

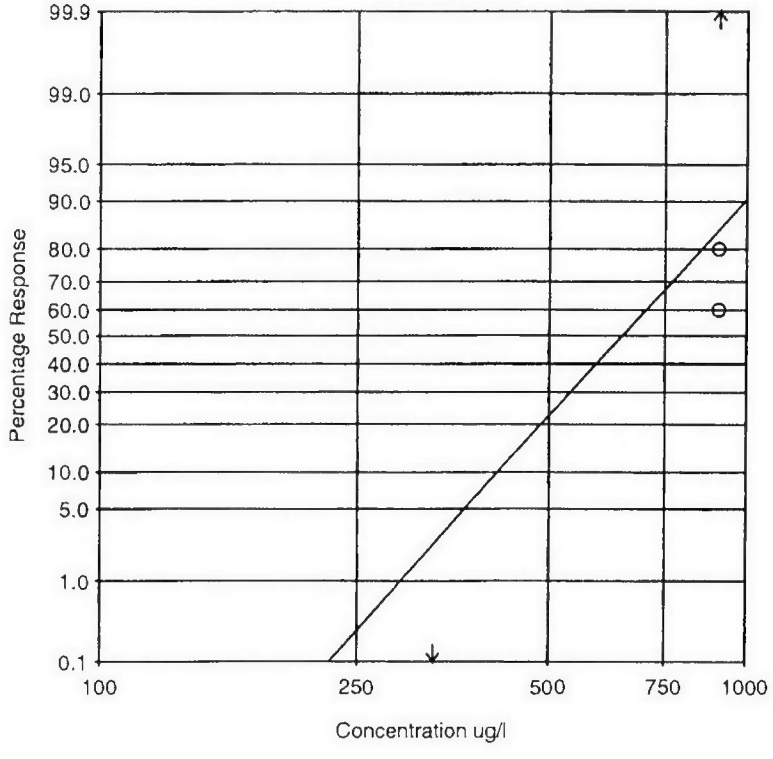


Figure 2 Percentage response (=immobility) of *Daphnia magna* as function of the log concentration of FR-1435X at 48h

APPENDIX II REFERENCE TEST

Start: 29 November 2004
End : 01 December 2004

48-hour Acute Toxicity Study in *Daphnia magna* with $K_2Cr_2O_7$ (NOTOX Project 425666).

The study procedures described in this report were based on the ISO International Standard 6341, the EEC directive 92/69, Part C.2. "Acute toxicity for *Daphnia*", December 1992, and the OECD guideline No. 202: "Daphnia sp., Acute Immobilisation Test", Adopted April 4, 1984.

The reference test was carried out to check the sensitivity of the test system as used by NOTOX. Daphnia were exposed for a maximum of 48 hours to $K_2Cr_2O_7$ concentrations of 0.10, 0.18, 0.32, 0.56, 1.0 and 1.8 mg/l and to a blank control. Twenty daphnia were exposed per concentration.

The reference substance, potassium dichromate ($K_2Cr_2O_7$, art. 4864, batch no. K28974764) was obtained from Merck, Darmstadt, Germany.

Acute immobilization of daphnia after 24 and 48 hours in the reference test with potassium dichromate:

Concentration (mg/l)	Number Exposed	% immobile		Expected response (%) After 48 hours [1]	
		24h	48h	Minimal	Maximal
Blank-control	20	0	0	0	10[2]
0.10	20	0	0	0	10
0.18	20	0	5	0	10
0.32	20	0	5	0	30
0.56	20	15	80	0	100
1.0	20	90	100	40	100
1.8	20	100	100	100	100

[1] Based on historical data of the previous years (n>60).
[2] A maximum response of 10% does not invalidate the results of the test.

The actual responses in this reference test with $K_2Cr_2O_7$ are within the ranges of the expected responses at the different concentrations. Hence, the sensitivity of this batch of *D. magna* was in agreement with the historical data collected at NOTOX.

The 24h-EC_{50} was 0.69 mg/l with a 95% confidence interval between 0.61 and 0.81 mg/l.

The 48h-EC_{50} was 0.46 mg/l with a 95% confidence interval between 0.41 and 0.52 mg/l.

The raw data from this study are kept in the NOTOX archives. The test described above was performed under GLP with a QA-check.

APPENDIX III ANALYSES OF TAP WATER (Not performed under GLP)

Date of sampling : 10-11-04
Sample numbers : 1859354/1859355
Laboratory : ANALYTICO MILIEU B.V., Barneveld, The Netherlands

COMPONENT:	ANALYSIS:	
Metals:		
Selenium	< 0.90	µg/l
Arsenic	< 5.0	µg/l
Cadmium	< 0.40	µg/l
Chromium	< 1.0	µg/l
Copper	24	µg/l [a)]
Mercury	0.074	µg/l
Lead	<5.0	µg/l
Zinc	14	µg/l
Manganese	<0.010	mg/l
Calcium	40	mg/l [a)]
Magnesium	9.3	mg/l [a)]
Iron	<0.050	mg/l
EOX (extractable organic halogens)	<1.0	µg/l
Polycyclic Aromatic Hydrocarbons:		
Naphtalene	0.073	µg/l
Acenaphtylene	0.062	µg/l
Acenaphtene	< 0.010	µg/l
Fluorene	< 0.010	µg/l
Phenanthrene	< 0.010	µg/l
Anthracene	< 0.0050	µg/l
Fluoranthene	< 0.010	µg/l
Pyrene	< 0.010	µg/l
Benzo(a)antracene	< 0.010	µg/l
Chrysene	< 0.010	µg/l
Benzo(b)fluoranthene	< 0.010	µg/l
Benzo(k)fluoranthene	< 0.010	µg/l
Benzo(a)pyrene	< 0.010	µg/l
Dibenzo(ah)antracene	< 0.010	µg/l
Benzo(ghi)perylene	< 0.010	µg/l
Indeno(123cd)pyrene	< 0.010	µg/l
PAC's Total EPA (16)	0.13	
PAC's Total VROM (10)	0.073	

Total aerobic germ-count (22ºC)	34	CFU/ml [a)]
Total aerobic germ-count (37ºC)	< 1	CFU/ml [a)]
Hardness (expr. as Ca + Mg):		1.2 mmol/l [a)]
Nitrate (expr. as N):	0.71	mg/l
Nitrite (expr. as N):	<0.01	mg/l
Cyanide-totaal (NEN 6655)	<1.0	ug/l
Turbidity	0.21	FTE
Colour intensity	21	mg Pt/l

Dechlorination of tap water was not necessary since no chlorine was present.

[a)] Tap water was sampled from the copper-free water supply system that supplied the water used for the present toxicity study. Among others, copper and bacterial activity were measured in these samples. The other components were analysed in samples taken from the same water supply but sampled in the Animal House.

ORIGINAL

PROTOCOL AMENDMENT NO : 1

Study Title	Acute toxicity study in *Daphnia Magna* with FR-1435X (static)
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419816

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

1. Page 6, paragraph 4.1.1. Test substance information:

Description	Brown liquid

REASONS FOR AMENDMENT

1-2 The data has been updated according to information of the sponsor, dated January 06, 2005.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

12 January 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title Acute toxicity study in *Daphnia Magna* with FR-1435X (static)

Sponsor Bromine Compound Ltd.
P.O. Box 180
BEER-SHEVA 84101
Israel

Study Monitor Dr. S. Lifshitz

NOTOX Substance 146232/A

NOTOX Project 419816

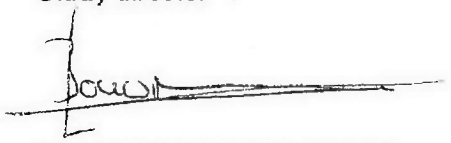
AMENDMENT DESCRIPTION

1. The final test will be performed applying a flow-through system with continuous renewal of test solutions with the following test conditions:
   - Target concentrations: 0.05, 0.10, 0.20, 0.50 and 1.1 mg/l.
   - Test medium: Tap-water (Hardness 200-220 mg $CaCO_3$/l).
   - Stock solution(s) will be prepared in acetone and dosed via a computer-controlled system connected to a dispenser (Gilson). Via this system the dosed volume enters a mixing flask separately from the tap water supply. The tap water will be supplied via a flow meter. The flow rate will be between 6 and 12 l/h. In the mixing flask the dosed volume and the tap water will be mixed under continuous stirring. The whole system will be checked daily.
   - Daphnia test vessel: A stainless steel vessel (ca. 1.5 litres) containing 4 mesh containers of stainless steel, covered by a removable glass or Perspex plate for the exposure of daphnia.
   - Before introduction of the organisms, test concentrations will be allowed to stabilise for at least 12 hours after the start of the dosing.
   - Samples for analysis will be taken one day before the start of exposure (check functioning system), at the start and after 48 hours from the control and all target concentrations.
2. The final test will be performed in week 8. The proposed reporting date will be 10 April 2005.

REASONS FOR AMENDMENT

1 and 2: Analytical results obtained during the range-finding test with carp showed that measured concentrations could not be maintained stable during the test period. During the first 24-hour test period a decrease of more than 20% was observed at nominally 0.05 and 5.0 mg/l. Consequently, test procedures need to be changed to flow-through with continuous renewal to maintain stable concentrations.

APPROVAL
Study director



Ir. L.M. Bouwman

01 February 2005
date:

Sponsor

Sarit Lifshitz
Dr. S. Lifshitz

6/2/05
date:

PROTOCOL AMENDMENT NO : 3

ORIGINAL

Study Title	Acute toxicity study in *Daphnia Magna* with FR-1435X (static)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419816

AMENDMENT DESCRIPTION

During the final flow-through test duplicate or triplicate samples will be taken from the control and all target concentrations.

REASONS FOR AMENDMENT

Duplicate or triplicate samples are required for the analytical validation.

APPROVAL

Study director

.....
Ir. L.M. Bouwman

03 February 2005
.....
date:

Sponsor

Sarit Lifshitz
.....
Dr. S. Lifshitz

6/2/05
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 4

Study Title	Acute toxicity study in *Daphnia Magna* with FR-1435X (static)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419816

AMENDMENT DESCRIPTION

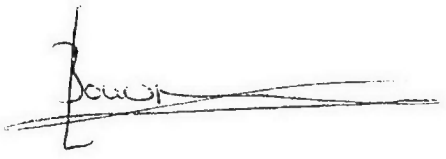
1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director



10 February 2005

.....
Ir. L.M. Bouwman

.....
date:

PROTOCOL AMENDMENT NO : 5

ORIGINAL

Study Title	Acute toxicity study in *Daphnia Magna* with FR-1435X (static)
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419816

AMENDMENT DESCRIPTION

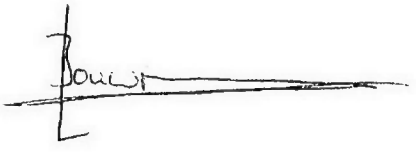
1. The EEC guideline should read:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.2. "Acute Toxicity for *Daphnia*".

REASONS FOR AMENDMENT

1. Correction of the text in the protocol.

APPROVAL
Study director



07 April 2005

....

Ir. L.M. Bouwman date:

PROTOCOL AMENDMENT NO : 6

ORIGINAL

Study Title	Acute toxicity study in *Daphnia Magna* with FR-1435X (static)
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419816

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1. Test substance information:

Density	~2000 kg/m^3

REASONS FOR AMENDMENT

1. The data has been added according to information of the sponsor, dated 15 August 2005
   This has no effect on other data.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

26 August 2005
.....
date:

4. SUMMARY

A high performance liquid chromatographic (HPLC) method for quantitative analysis of the test substance (FR-1435X) in ISO-medium was implemented. A Zorbax RX-C18 column was used with 90/10 (v/v) Methanol/Milli-Q water as the mobile phase and a spectrophotometric detector set to read the absorbance at 225 nm.

Standard solutions of the test substance were prepared in Methanol at exactly known concentrations between 860 mg/l and 1082 mg/l, ultrasonicated for at least 15 minutes to dissolve the test substance completely and diluted with Methanol. Calibration solutions for the validation tests were obtained by further diluting these dilutions in a 1:1 (v:v) ratio with ISO-medium. End solution for all samples was 50/50 (v/v) Methanol/ISO-medium. Accuracy samples were prepared using a 986 or 1016 mg/l test substance solution in Methanol.

The method was validated for the following parameters:

4.1. Specificity

Two test substance peaks were observed in chromatograms of test substance solutions. It was assumed that these peaks derive from the major components in the test substance. The area of the sum of the test substance peak was used as test substance response in the calculations during the validation tests. The chromatogram of a blank solution showed no peak with the same retention time as that of the major components of the test substance.

Since no interferences were detected in blank chromatograms, the specificity requirements were met and the analytical method was found to be specific for the test substance.

4.2. Linearity

A linear relationship between response and test substance concentration was found over a concentration range of 0.00601 – 0.601 mg/l (in end solution) using a ($1/concentration^2$) weighting factor. Since the correlation coefficient (r) of the weighted regression is > 0.990, the calibration line was accepted.

4.3. Accuracy

A blank solution (ISO-medium; 10 ml) was spiked in five-fold at concentration levels of 0.0251, 0.503, 0.996 and 5.03 mg/l. The 5.03 mg/l accuracy samples were ultrasonicated for 5 minutes. To all aqueous samples, 10 ml Methanol was added. In this way, a dilution factor of 2 was obtained. The 5.03 mg/l accuracy samples were further diluted by a factor of 10 with 50/50 (v/v) Methanol/ ISO-medium, resulting in a dilution factor of 20. At all concentration levels, mean recoveries between 70% and 110% were obtained. Therefore, the analytical method was considered applicable to samples in ISO-medium in the concentration range 0.0251 mg/l to 5.03 mg/l.

4.4. Repeatability

Based on the results from the accuracy samples, it was concluded that at all concentration levels the coefficient of variation was < 20%. Therefore, the method was found to be applicable for analysis of samples in ISO-medium in the concentration range of 0.0251 – 5.03 mg/l.

4.5. Limit of Quantification (LOQ)

Based on the results obtained during this project, the LOQ was determined to be 0.0251 mg/l in ISO-medium.

4.6. Limit of Detection (LOD)

The limit of detection for the test substance was determined to be 0.0021 mg/l (in end solution) at an injection volume of 100 µl. Taking a dilution factor of 2 into account, this corresponds to a limit of detection of 0.0042 mg/l in ISO-medium.

4.7. Stability of the Chromatographic System and End Solutions

The chromatographic system and end solutions containing the test substance 0.00601 and 0.601 mg/l) were stable for at least 15.5 hours when stored at room temperature in the dark.

4.8. Stability of Standard Solutions

Standard solutions (860 mg/l and 1040 mg/l) of the test substance in Methanol were stable for at least 168 hours when stored at room temperature.

4.9. Storage Stability of Samples

The test substance was found to be stable in ISO-medium in the concentration range 0.0254 to 5.08 mg/l during storage in the freezer for 18 hours.

- Attachment 16 -

REPORT

Study Title

96-HOUR ACUTE TOXICITY STUDY IN CARP WITH FR-1435X (FLOW-THROUGH)

Author

Ir. L.M. Bouwman

Study completion date

30 August 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419805
NOTOX Substance 146232/A

1. CONTENTS

TABLES

FIGURES

APPENDICES

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

Tap water analyses were not performed under GLP and therefore excluded from this GLP statement.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: 30 August 2005

Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology

Date: 31/08/2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	protocol	14-Oct-04	14-Oct-04	14-Oct-04
	Amendment 1 of protocol	12-Jan-05	12-Jan-05	12-Jan-05
	Amendment 2 of protocol	02-Feb-05	02-Feb-05	02-Feb-05
	Amendment 3 of protocol	04-Feb-05	04-Feb-05	04-Feb-05
	Amendment 4 of protocol	11-Feb-05	11-Feb-05	11-Feb-05
	Amendment 5 of protocol	08-Apr-05	08-Apr-05	08-Apr-05
	Report	11-Apr-05	11-Apr-05	12-Apr-05
	Amendment 6 of protocol	26-Aug-05	26-Aug-05	26-Aug-05
Process	**Ecotoxicology & Biodegradation** Test substance handling Exposure Observations/Measurements	17-Jan-05	21-Jan-05	21-Jan-05
	Analytical and physical chemistry Test substance handling Observations/Measurements	10-Jan-05	14-Jan-05	20-Jan-05

Head of Quality Assurance
C.J.Mitchell B.Sc.

Date: 31/8/05

4. SUMMARY

96-Hour Acute Toxicity Study in Carp with FR-1435X.

The study procedure described in this report was based on the ISO International Standards 7346-1 and 7346-3: Static method and Flow-through method, 1996. In addition, the procedures were designed to meet the test methods and validity criteria of the EEC directive 92/69, Part C.1, 1992, the OECD guideline No. 203, 1992 and the OECD series on testing and assessment number 23, 2000.

The batch of FR-1435X tested was a brown liquid with a purity of 97.2%. The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor). Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone.

The project started with a static range-finding test. Three fish per concentration were exposed to nominal FR-1435X concentrations of 0.05, 0.50 and 5.0 mg/l. Within 48 hours of exposure all fish exposed to the highest test concentration had died. No fish died at the two lower test concentrations during the total test period. The expected LC_{50} was between nominal 0.50 and 5.0 mg/l. The analytical results of the samples taken during the test showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.40 and 3.7 mg/l, respectively. The lowest and the highest actual test concentrations decreased by more than 20% over the first 24 hours of exposure, while the 0.50 mg/l concentration remained stable. During the last 72 hours all concentrations decreased. Hence, as 2 out of 3 concentrations decreased by more than 20% during the first 24-hour test period it was decided to continue testing applying a continuous renewal system for the final test.

The project was continued with a final test in a flow-through system. Target concentrations were 0.20, 0.50, 1.1, 2.4 and 5.0 mg/l. Seven fish per concentration were exposed per target concentration and a solvent-control for a maximum of 96 hours. The stock solutions in acetone were prepared daily and were dosed via a computer controlled system consisting of 6 dispensers (Gilson). Via this system the dosed volume entered a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter with a flow rate of ca. 12 l/h. In the mixing flask the dosed volume and the tap water were mixed under continuous stirring. Subsequently, the test solution coming from the mixing flask entered the vessel containing the fish. The dosing system was started two days before introduction of the organisms and the whole system was checked daily. Samples for analytical confirmation of actual test concentrations were taken 24 hours prior to the exposure period and at the start, after 48, 72, 92 hours of exposure and at the end of the test.

Analyses of the samples taken during the final test showed that the actual concentrations were generally in agreement with the target concentrations during the test period and stable within 20% of the start concentrations. Based on the results above, the LC_{50} and the NOEC were based on mean exposure concentrations: 0.17, 0.45, 1.0, 2.3 and 3.9 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

FR-1435X induced mild visible effects in carp at the lowest concentration tested. Therefore, the NOEC was < 0.17 mg/l.

The 96h-LC_{50} was 0.76 mg/l based on mean exposure concentrations (95% confidence interval between 0.66 and 1.0 mg/l). The 96h-LC_{50} was above the water solubility of FR-1435X.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	Ing. S. Jilesen
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start : 22 November 2004 Completion : 07 March 2005

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate acute toxic effects in *Cyprinus carpio* during an exposure period of 96 hours and, if possible, to determine the LC_{50} at all observation times.

5.3. Guidelines

The study procedure described in this report was based on the ISO International Standards 7346-1 and 7346-3: "Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]" – Part 1: Static method and Part 3: Flow-through method, Second edition, 1996-06-15.

In addition, the procedure is designed to meet the test methods and validity criteria prescribed by the following guidelines and guidance documents:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.1. "Acute toxicity for fish".
- The OECD guidelines for Testing of Chemicals, guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Fish were considered to be **dead** when no reaction was observed after touching the caudal peduncle and visible breathing movements were absent. In addition, fish that were convulsing or showing other severe forms of distress not considered transient in nature and likely to become more severe before the exposure is terminated, will be sacrificed for humane reasons. These fish will be treated as having died in the test, based on OECD guideline on humane endpoint (ENV/JM/MONO/(2000)7).

The **LC_{50}** is the concentration killing 50% of the fish after a defined period of exposure.

A **Flow-through test** is a test in which the water containing the test substance is renewed continuously.

6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Test substance

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000 kg/m^3
Stability in water	Not indicated

6.1.2. Reference substance

This report includes the results of a reference test with pentachlorophenol (PCP) (Appendix II).

6.1.3. Preliminary data

The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor). FR-1435X concentrations in the range of 0.0254 and 5.08 mg/l in ISO-medium showed to be stable during freezing and thawing of samples (NOTOX Project 419838).

6.2. Test System

Species	Carp (*Cyprinus carpio*, Teleostei, Cyprinidae) Linnaeus, 1758
Source	Zodiac, proefacc, "De Haar Vissen", L.U. Wageningen, the Netherlands.
Mean length	Static range-finding test: 2.8 ± 0.4 cm Flow-through final test: 2.4 ± 0.1 cm
Mean weight	Static range-finding test: 0.64 ± 0.28 g Flow-through final test: 0.40 ± 0.08 g
Characteristics	F1 from a single parent-pair bred in UV-treated water.
Reason for selection	This system has been selected as an internationally accepted species.
Total fish used	51

6.3. Holding

Quarantine/Acclimatisation	At least 12 days after delivery.
Medium	ISO-medium, formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$CaCl_2.2H_2O$	293.8	mg/l
$MgSO_4.7H_2O$	123.3	mg/l
$NaHCO_3$	64.8	mg/l
KCl	5.8	mg/l

Measurements	pH, nitrate and nitrite concentration and ammonia concentration: once a week. Temperature: every day.
Feeding	Daily with pelleted fish food and/or artemia.
Validity of batch	In the batch of fish used for the test, mortality during the seven days prior to the start of the test was less than 5%.

6.4. Static range-finding test

Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone (99.8%, Labscan, Ireland) at concentrations a factor of 10,000 above the nominal concentrations in test medium. Subsequently, amounts of 0.5 ml were added to 5 l ISO-medium to obtain the final test concentrations of 0.05, 0.50 and 5.0 mg/l. After a magnetic stirring period of 18 minutes the resulting solutions were clear and colourless, except for the 5.0 mg/l concentration that was slightly hazy.

Part of the test solutions was used for the simultaneously performed acute *Daphnia magna* toxicity test (NOTOX Project 419816).

A static range-finding test was performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those

applied in the final test with the following exceptions: Three fish per concentration were exposed to 0.05, 0.50 and 5.0 mg/l. Test solutions were not renewed during the test period. Dissolved oxygen concentrations, pH and temperature were only measured in the lowest and the highest test concentration with surviving fish. Aeration was introduced after 24 hours of exposure and was maintained until the end of the test. Samples for possible analysis were taken from all concentrations.

Sampling: Frequency	at t=0 h, t=24 h and t=96 h
Volume	700 µl
Storage	Samples were stored in a freezer until analysis.

6.5. Flow-through final test

6.5.1. Test concentrations

FR-1435X	0.20, 0.50, 1.1, 2.4 and 5.0 mg/l
Control	Test medium without test substance but containing acetone as used in the treatment of the stock solutions (solvent-control).

6.5.2. Test procedure and conditions

Test duration	96 hours
Test type	Flow-through, continuous renewal of test media
Test vessels	25 litres, all-glass
Test medium	Tap water (see Appendix III for data of analysis)
Number of fish	7 fish per concentration and control
Loading	0.01 g fish.litre^{-1}.day^{-1}
Illumination	16 hours photoperiod daily
Aeration	The test media were not aerated during the test.
Feeding	No feeding from 48 hours prior to the test and during the total test period.
Introduction of fish	Two days after the start of the dosing to permit stabilisation of the system.
Euthanasia	At the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.

6.5.3. Preparation of stock solutions

Due to the low water solubility of the test substance, stock solutions in acetone (99.8%, Labscan, Ireland) were prepared at concentrations a factor of 10,000 above the target concentrations in test medium. These stocks were used for dosing and were prepared daily.

6.5.4. Dosing system

The stock solutions in acetone were dosed via a computer controlled system consisting of 6 dispensers (Gilson). Via this system the dosed volume entered a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter with a flow rate of ca. 12 l/h. In the mixing flask the dosed volume and the tap water were mixed under continuous stirring. Subsequently, the test solution coming from the mixing flask entered the vessel containing the fish. The whole system was checked daily. The dosing program is described in Table 1 and a diagram of the system is presented in Figure 1.

Table 1 Dosing program

Stock solution FR-1435X in acetone (g/l)	Dosing volumes (1.0 ml syringe)		Target concentrations FR-1435X (mg/l)
	Per cycle ml/5 min	Per hour ml/hour	
2.0	0.10	1.2	0.20
5.0	0.10	1.2	0.50
11	0.10	1.2	1.1
24	0.10	1.2	2.4
50	0.10	1.2	5.0

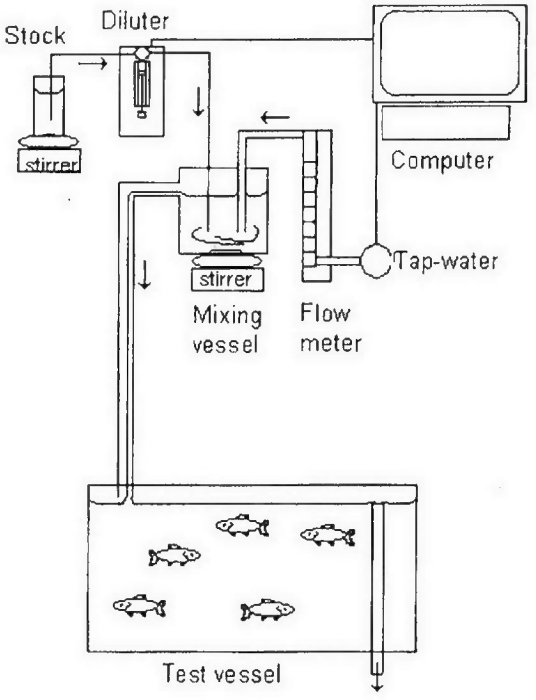


Figure 1 Flow-through system

6.5.5. Sampling for analysis of test concentrations

During the final test duplicate samples were taken from concentrations < 1.0 mg/l and triplicate samples were taken from concentrations ≥ 1.0 mg/l. These samples were used for analyses and were taken according to the schedule below. The method of analysis is described in the appended Analytical Report (Appendix V).

Sampling: Frequency	24 hours following the start of dosing, samples were taken from all target concentrations and the control to check the actual exposure concentration. At the start, after 48 hours of exposure and the end of the exposure period samples were taken from all target concentrations with surviving fish and the control. In addition, samples were taken from the highest target concentration after 72 hours of exposure and from the 2.4 mg/l target concentration at 92 hours of exposure.
Volume	10 ml
Storage	Samples taken for system check, at the start, after 92 hours and at the end of the test were analysed on the day of sampling. Samples taken after 48 and 72 hours of exposure were directly diluted with methanol (1:1) and stored at room temperature until analysis.

6.5.6. Measurements and recordings

Mortality and other effects	At 2½, 24, 48, 72 and 96 hours following the start of exposure. Dead fish were removed when observed.
Fish length and weight	Ten fish of the batch used for the test, were weighed and measured prior to the start of the test.
Dissolved oxygen content, pH and temperature	Daily in all vessels, beginning at the start of the test (day 0).

6.6. Interpretation

6.6.1. Data handling

Defining exposure concentrations:

The results were based on the mean of the concentrations measured at the various sampling intervals during the exposure period.

The LC_{50} was determined using the maximum likelihood estimation method with the probits of the percentages of dead fish as function of the logarithms of the corresponding concentrations (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition).

6.6.2. Acceptability of the test

1. No mortality or abnormal behaviour was observed in the control group.
2. The oxygen concentration was maintained at at least 60% of the air saturation value throughout the test (> 5 mg/l at 22 °C). Other test conditions (pH and temperature) were maintained within the limits prescribed by the guidelines.
3. The analytical program provided clear evidence that all actual test concentrations were maintained within a maximum variation of ±20% during the test period.
4. The 96h-LC_{50} of the reference chemical for the stock of fish was in agreement with results obtained previously in our laboratory.

6.7. List of protocol deviations

1. For the final test 25 litre vessels were used.
   Evaluation: By using smaller test vessels, the total volume change of the test vessels per time unit was enlarged.

The study integrity was not adversely affected by this deviation.

7. RESULTS

7.1. Static range-finding test

7.1.1. Mortality and other effects

The results of the range-finding test are presented in Table 2. After 48 hours of exposure all fish exposed to the highest test concentration had died. No fish died at the two lower test concentrations during the total test period. The expected LC_{50} was between nominal 0.50 and 5.0 mg/l.

All test conditions were maintained within the limits prescribed by the protocol.

Table 2 Incidence of mortality and total mortality during the static range-finding test

Nominal conc. FR-1435X (mg/l)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		3½h	24h	48h	72h	96h	
0.05	3	0	0	0	0	0	0
0.5	3	0	0	0	0[1]	0[1]	0
5.0	3	0[1]	0[2]	3	3	3	100

[1] All three fish were hypoactive
[2] One fish was hypoactive, one fish was hypoactive and showed loss of equilibrium, one fish was immobile.

7.1.2. Stability of FR-1435X under test conditions

The analytical results showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.40 and 3.7 mg/l, respectively. The lowest and the highest actual test concentrations decreased by more than 20% over the first 24 hours of exposure, while the 0.50 mg/l concentration remained stable. During the last 72 hours all concentrations decreased (see Table 2 of the appended Analytical Report). Hence, as 2 out of 3 concentrations decreased by more than 20% during the first 24-hour test period it was decided to continue testing applying a continuous renewal system for the final test.

7.2. Flow-through final test

7.2.1. Measured concentrations

The results of analysis of the samples taken during the final test are described in Table 3 of the appended Analytical Report. Analyses showed that the actual concentrations were generally in agreement with the target concentrations during the test period and stable within 20% of the start concentrations. Based on the results above, the LC_{50} and the NOEC were based on mean exposure concentrations: 0.17, 0.45, 1.0, 2.3 and 3.9 mg/l.

7.2.2. Mortality and other effects

Table 3 shows the mortality data recorded during the flow-through final test. The responses recorded in this test allowed for reliable determination of an LC_{50}. Table 3 specifies the clinical effects observed at different test concentrations.

Table 3 Incidence of mortality and total mortality during the flow-through final test

Mean conc. FR-1435X (mg/l)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		2½h	24h	48h	72h	96h	
Solvent-control	7	0	0	0	0	0	0
0.17	7	0	0	0	0	0	0
0.45	7	0	0	0	0	0	0
1.0	7	0	0	3	4	6	86
2.3	7	0	0	6	6	7	100
3.9	7	0	0	6	7	7	100

Table 4 Clinical effects observed during the flow-through final test.

Mean conc. FR-1435X (mg/l)	Time of recording (hours)	Specification of effects	Relative number
Control	3½-96	No abnormalities	7/7
0.17, 0.45	3½	No abnormalities	7/7
	24-96	Hypoactive swimming	7/7
1.0	3½	No abnormalities	7/7
	24	Hypoactive swimming at the bottom	5/7
		Immobile	2/7
	48	Swimming at the bottom	3/4
		Immobile/residing at the bottom	1/4
	72	Immobile/residing at the bottom	3/3
	96	Immobile	1/1
2.3	3½	No abnormalities	7/7
	24	Immobile	6/7
		Hypoactive swimming	1/7
	48-72	Immobile/residing at the bottom	1/1
3.9			
	3½	Hypoactive swimming	7/7
	24	Immobile	7/7
	48	Immobile/residing at the bottom	1/1

7.2.3. Determination of effect concentrations

Table 5 shows the effect parameters based on mean concentrations, see also Appendix I.

Table 5 Effect parameters

Parameter	Concentration FR-1435X (mg/l)	95%-confidence interval
NOEC	< 0.17	
48h-LC_{50}	1.3	0.81 - 2.2
72h-LC_{50}	1.1	0.72 - 1.7
96h-LC_{50}	0.76	0.66 - 1.0

7.2.4. Experimental conditions

The results of measurement of pH and oxygen concentrations are presented in Table 6. The temperatures measured during the study in the various test vessels are presented in Table 7. All test conditions remained within the ranges prescribed by the protocol (pH: 6.0-8.5, constant within 1 unit; temperature 20-24°C, constant within 2°C; oxygen > 60% of air saturation).

Table 6 pH-values and dissolved oxygen concentrations (mg/l) during the flow-through final test

Mean conc. FR-1435X (mg/l)	Day 0		Day 1		Day 2		Day 3		Day 4	
	pH	O_2	pH	O_2	pH	O_2	pH	O_2	pH	O_2
Solvent-control	8.0	9.5	8.0	10.2	8.0	9.1	8.0	9.2	7.9	9.6
0.17	8.0	9.4	7.9	10.2	7.9	9.2	7.9	9.1	7.9	9.3
0.45	7.9	9.6	7.8	10.6	7.9	9.3	7.9	9.2	7.9	9.4
1.0	7.9	9.7	7.9	10.4	7.9	9.2	7.9	9.2	7.8	9.2
2.3	7.9	9.8	7.8	10.4	7.9	9.1	7.9	9.2	7.8	9.1
3.9	7.9	9.9	7.9	10.4	7.9	9.1	7.9	9.2		

Table 7 Temperatures (°C) measured during the flow-through final test

Mean conc. FR-1435X (mg/l)	Day 0	Day 1	Day 2	Day 3	Day 4
Solvent-control	20.7	20.9	20.8	20.7	20.6
0.20	20.7	20.9	20.9	20.9	20.9
0.44	20.7	21.0	21.0	21.0	21.0
0.62	20.7	21.0	21.0	21.0	21.0
0.98	20.8	21.1	20.5	21.0	20.9
1.6	20.7	20.9	20.8	21.0	

8. CONCLUSION

Under the conditions of the present test FR-1435X induced mild visible effects in carp at the lowest concentration tested. Therefore, the NOEC was < 0.17 mg/l.

The 96h-LC_{50} was 0.76 mg/l based on mean exposure concentrations (95% confidence interval between 0.66 and 1.0 mg/l). The 96h-LC_{50} was above the water solubility of FR-1435X.

APPENDIX I LC-VALUES

Table 8 48h-LC_{50} values and related parameters

48h-LC_{50} = 1345.2 µg/l
95 % fiducial limits: 808.2 - 2164.2 µg/l

index of regression significance: g=0.32
chi-squared=1.57, with 2 degrees of freedom

regression line: log10(conc.)=3.14+(probit-5.05)/3.25

conc. µg/l	group size	mortality	corrected fraction	expected fraction	chi2
450	7	0	0.00	0.05	0.39
1000	7	3	0.43	0.34	0.25
2300	7	6	0.86	0.78	0.26
3900	7	6	0.86	0.93	0.67
					1.57

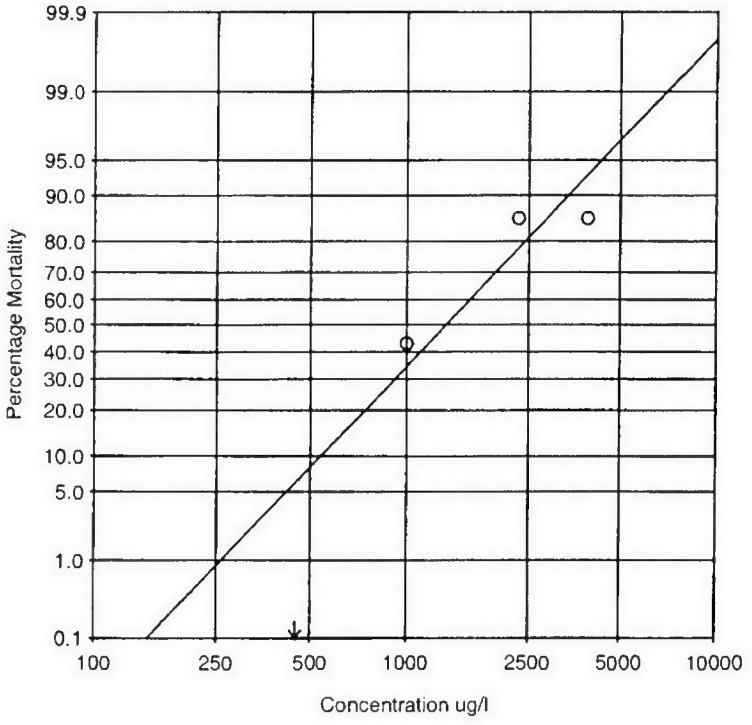


Figure 2 Percentage of mortality of fish plotted against the concentration after 48 hours of exposure

APPENDIX I LC-VALUES - continued -

Table 9 72h-LC_{50} values and related parameters

$72h\text{-}LC_{50}$ = 1087.0 µg/l
95 % fiducial limits: 722.3 - 1656.7 µg/l

index of regression significance: g=0.31
chi-squared=1.23, with 2 degrees of freedom

regression line: log10(conc.)=3.03+(probit-4.97)/4.36

conc. µg/l	group size	mortality	corrected fraction	expected fraction	chi2
450	7	0	0.00	0.03	0.25
1000	7	4	0.57	0.44	0.51
2300	7	6	0.86	0.92	0.42
3900	7	7	1.00	0.99	0.05
					1.23

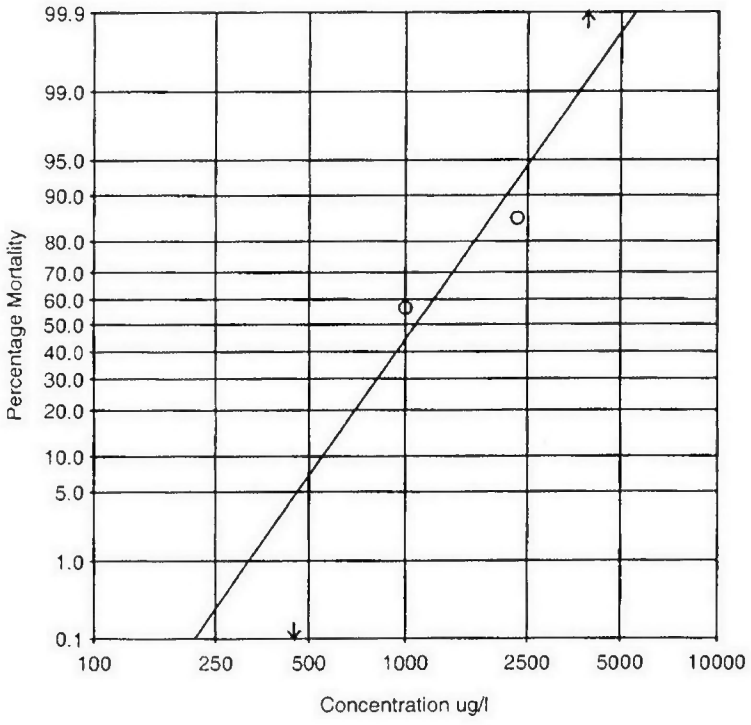


Figure 3 Percentage of mortality of fish plotted against the concentration after 72 hours of exposure

APPENDIX I LC-VALUES - continued -

Table 10 96h-LC_{50} values and related parameters

$96h\text{-}LC_{50}$ = 757.7 µg/l
95 % fiducial limits: 657.4 - 1034.7 µg/l

index of regression significance: g=0.14
chi-squared=0.00, with 1 degrees of freedom

regression line: log10(conc.)=2.65+(probit-3.01)/8.84

conc. µg/l	group size	mortality	corrected fraction	expected fraction	chi2
450	7	0	0.00	0.00	0.00
1000	7	6	0.86	0.86	0.00
2300	7	7	1.00	1.00	0.00
					0.00

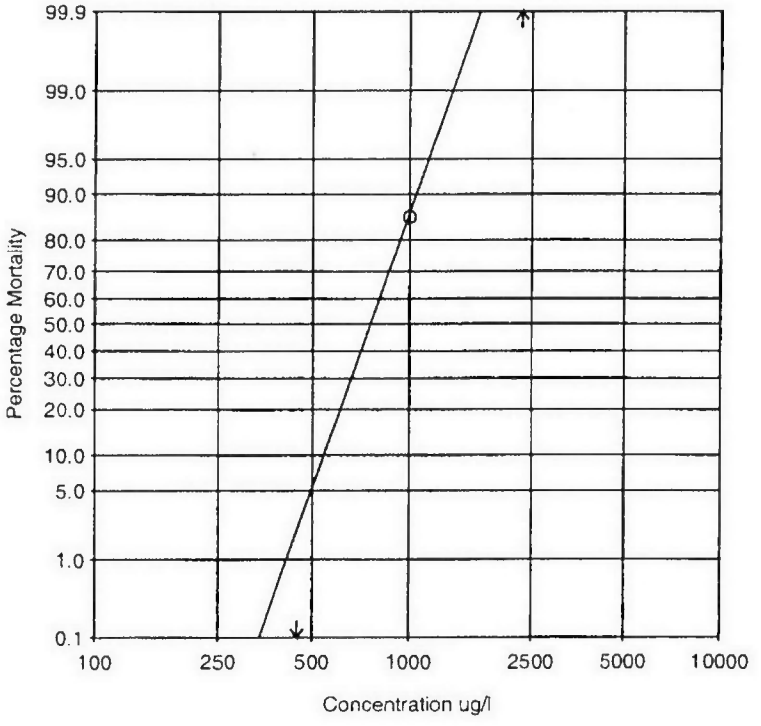


Figure 4 Percentage of mortality of fish plotted against the concentration after 96 hours of exposure

APPENDIX II REFERENCE TEST

96-hour acute toxicity study in the carp with PCP; NOTOX Project 430662 (Batch K04-17)

The study procedures described in this report were based on the EEC directive 92/69, Part C.1, 1992 "Acute toxicity for fish"; and the OECD guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

Start: 17 January 2005
End: 21 January 2005

This reference test was carried out to check the sensitivity of the test system as used by NOTOX. The reference substance was pentachlorophenol (PCP, SIGMA, Art. P9441, Batch 103H3488).

Concentrations: 0.10, 0.22 and 0.46 mg/l in ISO-medium.

Incidence of mortality observed in the reference study:

Concentration PCP (mg/l) Nominal	Initial number of fish	Cumulative number of dead fish recorded at various time points after start of exposure					Total Mortality (%)
		2¼h	24h	48h	72h	96h	
0.10	5	0	0	0	0	0	0
0.22	5	0	5	5	5	5	100
0.46	5	0	5	5	5	5	100

During the test the pH, oxygen concentration and the temperature of the medium were within the optimal ranges for fish.

Under the conditions of the present test PCP induced no lethal effects in carp at 0.10 mg/l. The 96h-LC_{50} for carp exposed to PCP was 0.15 mg/l (a 95% confidence interval between 0.10 and 0.22 mg/l) and was already reached within 24 hours of exposure. The 96h-LC_{50} for carp is generally between 0.10 and 0.46 mg/l based on historical data of reference tests performed approximately every 3 months from April 1988 until the end 2000, and annually since then. Hence, the sensitivity of carp originating from the present batch for PCP falls within the range of sensitivities generally observed during the past years.

The raw data and report from this study are kept in the NOTOX archives. The test described above was performed under GLP-conditions with a QA-check.

APPENDIX III ANALYSES OF TAP WATER (Not performed under GLP)

Date of sampling : 10-11-04
Sample numbers : 1859354/1859355
Laboratory : ANALYTICO MILIEU B.V., Barneveld, The Netherlands

COMPONENT:	ANALYSIS:	
Metals:		
Selenium	< 0.90	µg/l
Arsenic	< 5.0	µg/l
Cadmium	< 0.40	µg/l
Chromium	< 1.0	µg/l
Copper	24	µg/l [a)]
Mercury	0.074	µg/l
Lead	<5.0	µg/l
Zinc	14	µg/l
Manganese	<0.010	mg/l
Calcium	40	mg/l [a)]
Magnesium	9.3	mg/l [a)]
Iron	<0.050	mg/l
EOX (extractable organic halogens)	<1.0	µg/l
Polycyclic Aromatic Hydrocarbons:		
Naphtalene	0.073	µg/l
Acenaphtylene	0.062	µg/l
Acenaphtene	< 0.010	µg/l
Fluorene	< 0.010	µg/l
Phenanthrene	< 0.010	µg/l
Anthracene	< 0.0050	µg/l
Fluoranthene	< 0.010	µg/l
Pyrene	< 0.010	µg/l
Benzo(a)antracene	< 0.010	µg/l
Chrysene	< 0.010	µg/l
Benzo(b)fluoranthene	< 0.010	µg/l
Benzo(k)fluoranthene	< 0.010	µg/l
Benzo(a)pyrene	< 0.010	µg/l
Dibenzo(ah)antracene	< 0.010	µg/l
Benzo(ghi)perylene	< 0.010	µg/l
Indeno(123cd)pyrene	< 0.010	µg/l
PAC's Total EPA (16)	0.13	
PAC's Total VROM (10)	0.073	

Total aerobic germ-count (22ºC)	34	CFU/ml [a)]
Total aerobic germ-count (37ºC)	< 1	CFU/ml [a)]
Hardness (expr. as Ca + Mg):	1.2	mmol/l [a)]
Nitrate (expr. as N):	0.71	mg/l
Nitrite (expr. as N):	<0.01	mg/l
Cyanide-totaal (NEN 6655)	<1.0	ug/l
Turbidity	0.21	FTE
Colour intensity	21	mg Pt/l

Dechlorination of tap water was not necessary since no chlorine was present.

[a)] Tap water was sampled from the copper-free water supply system that supplied the water used for the present toxicity study. Among others, copper and bacterial activity were measured in these samples. The other components were analysed in samples taken from the same water supply but sampled in the Animal House.

APPENDIX IV CERTIFICATE OF ANALYSIS

IMI (TAMI) Institute for Research & Development Ltd
Flame Retardants Department
P.O.Box 10140, Haifa Bay 26111, Israel Tel.+972-4-8469553
Fax:+972-4-846-9320 E-mail:zilbermanj@tami-imi.co.il

תמי (אימי) מכון למחקר ולפתוח בע"מ
מח' מעכבי בעירה
ת.ד. 10140, מפרץ חיפה 26111 טל. 04-8469553
פקס: 04-8469320 E-mail: zilbermanj@tami-imi.co.il

To: Lilach Yakobovich - DSBG

From: Y. Zilberman - IMI

Date: 14 December 2003

Following are some of the characteristics of the products.

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APPENDIX V

96-HOUR ACUTE TOXICITY STUDY IN CARP WITH FR-1435X (FLOW-THROUGH); DETERMINATION OF THE CONCENTRATIONS

Author

Dr. Ir. C. Brands

Study completion date

August 30, 2005

Laboratory Project Identification

NOTOX Project 419805
NOTOX Substance 146232/A

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FIGURES

2. REPORT APPROVAL

PRINCIPAL SCIENTIST: Dr. Ir. C. Brands
(Analytical Chemistry)

C Brands

.....

Date: August 30, 2005

3. INTRODUCTION

3.1. Preface

Study plan (analytical study)	Start	: 26 November 2004
	Completion	: 08 March 2005

3.2. Aim of the study

The purpose of the analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Methanol	HPLC-grade, Labscan, Dublin, Ireland
Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore Corp, Bedford, MA, USA
ISO-medium	see main report
Tap water	see main report

4.2. Samples

Static range-finding test

All samples were stored frozen. Stability of the samples under these conditions was determined during NOTOX Project 419838 "Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium". On the day of analysis, the frozen samples were defrosted at room temperature.

The samples (700 µl) were diluted in a 1:1 (v:v) ratio with methanol and thereafter ultrasonicated for 5 minutes. If necessary, the solutions were further diluted with 50/50 (v/v) methanol/ ISO-medium to obtain concentrations within the calibration range.

Flow-through final test

All samples were treated on the day of sampling and stored at ambient temperature if not analysed on the same day as sampling.

The samples (10 ml) were diluted in a 1:1 (v:v) ratio with methanol and thereafter ultrasonicated for 5 minutes. If necessary, the solutions were further diluted on the day of analysis with 50/50 (v/v) methanol/tap water to obtain concentrations within the calibration range.

4.3. Analysis

4.3.1. Analytical method

Quantitative analysis was based on the sum of both peaks in the HPLC chromatogram of FR-1435X (See NOTOX Project 419838: "Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium").

Analytical conditions

Column	
Stationary phase	Zorbax RX-C18
Dimensions	250 x 4.6 mm; dp = 5 µm
Brand	Agilent, Palo Alto, CA, USA
Mobile phase	90/10 (v/v) methanol/Milli-Q water
Flow	1.2 ml/min
Detection	Spectrophotometric (wavelength of 225 nm)
Injection volume	100 µl

4.3.2. Calibration

During the range-finding test, calibration solutions in 50/50 (v/v) methanol/ISO-medium were made up from two standard solutions of FR-1435X in methanol. On the first day of analysis of the final test, calibration solutions in 50/50 (v/v) methanol/tap water were made up from two standard solutions of FR-1435X in methanol. Standard solutions were ultrasonicated for 15 minutes in order to dissolve the test substance.

4.3.3. Injections

Calibration solutions were injected in duplicate. Procedural recovery samples and test samples were analysed by single injection.

4.3.4. Data acquisition and data processing

Data acquisition and data processing was performed using Empower software (Waters, Milford, MA, USA) version 5.00.

4.3.5. Procedural recovery samples

Standard solutions in methanol and dilutions of these solutions with methanol were used as spiking solutions. Standard solutions were ultrasonicated for 15 minutes in order to dissolve the test substance.

During the range-finding test and during the final test, blank test medium (10 ml) was spiked at concentration levels of approximately 0.025 mg/l, 0.5 mg/l and 5 mg/l [1] using spiking solutions with concentrations between 2.50 and 718 mg/l. At all concentration levels, samples were prepared and treated as described in paragraph 4.2 'Samples' (i.e. diluted in a 1:1 (v:v) ratio with methanol) and analysed. The recovery was calculated for each sample.

[1] Concentrations were not corrected for the spiking volume.

4.4. Interpretation

4.4.1. Calculations

All calculations were performed using non-rounded values. Rounded values are reported in the tables. Therefore, some differences might be observed when calculating the parameters as mentioned in the tables.

4.4.2. Formulas

General

Mean:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

where
x_i = measured value
n = number of measurements

Standard deviation

$$s_{n-1} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$$

Coefficient of variation

$$\frac{s_{n-1}}{\bar{x}} * 100\%$$

Calibration

Response: Peak area of test substance Peak 1 and Peak 2 [units]

Calibration curve: The response was correlated with the test substance concentration, using linear regression analysis (least squares method; weighting factor (1/concentration^2)).

$$R = a * C + b$$

R = response calibration solution [units]
C = concentration of test substance in calibration solution [mg/l]
a = slope [units*l/mg]
b = intercept [units]

Samples

Concentration of FR-1435X analysed in the samples:

$$C = \frac{(R - b) * d}{a} \quad [mg/l]$$

R = response sample [units]
d = dilution factor
a = slope [units*l/mg]
b = intercept [units]

Recovery of procedural recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100\ [\%]$$

Relative to nominal concentration:

$$\frac{\text{Concentration analysed}}{\text{Concentration nominal}} * 100\ [\%]$$

Relative to target concentration:

$$\frac{\text{Concentration analysed}}{\text{Concentration target}} * 100\ [\%]$$

Relative to initial concentration:

$$\frac{\text{Concentration analysed at t = x hours}}{\text{Mean concentration analysed at t = 0 h}} * 100\ [\%]$$

5. RESULTS

5.1. Calibration curves

For each series of analysis, a calibration curve was constructed using four concentration levels. During the range-finding test one data point was excluded. For the remaining concentration levels, two responses were used. During the final test, two responses were used at each concentration level. The coefficient of correlation was in excess of 0.99 for each calibration curve.

Figure 1 shows chromatograms of a calibration solution and blank 50/50 (v/v) methanol/tap water.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are summarized in Table 1. Figure 2 shows chromatograms of procedural recovery samples.

During the range-finding test, relatively high mean recoveries were determined (between 109% and 112%), which might indicate that concentrations analysed in the test samples are slightly overestimated. During the final test mean recoveries were between 92% and 109%, which indicated that analysis was performed properly.

5.2.2. Test samples

The results for the test samples are summarized in Tables 2 – 3. Figures 3 – 5 show chromatograms of solvent-control samples, target 0.20 mg/l samples and target 5.0 mg/l samples.

TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration nominal [mg/l]	Concentration analysed [mg/l]	Recovery [%]	Mean recovery (Coefficient of variation) [%]
29-11-04	30-11-04 [1]	0.0250	0.0276	110	112
		0.0250	0.0283	113	
		0.500	0.557	112	109
		0.500	0.536	107	
		5.00	5.54	111	110
		5.00	5.42	108	(1.2)
		5.00	5.53	111	
02-03-05	02-03-05	0.0251	0.0242	96	99
		0.0251	0.0256	102	
		0.503	0.539	107	107
		0.503	0.539	107	
		5.03	5.19	103	103
		5.03	5.24	104	(0.79)
		5.03	5.16	103	
03-03-05	03-03-05	0.0251	0.0227	90	92
		0.0251	0.0237	94	
		0.503	0.541	108	107
		0.503	0.534	106	
		5.03	5.15	102	102
		5.03	5.09	101	(0.79)
		5.03	5.07	101	
07-03-05	07-03-05	0.0251	0.0229	91	93
		0.0251	0.0240	96	
		0.503	0.540	107	109
		0.503	0.561	112	
		5.03	5.14	102	102
		5.03	5.12	102	(0.20)
		5.03	5.12	102	

[1] Procedural recovery samples and calibration solutions were prepared and treated on 29-11-04 and stored at ambient temperature until analysis on 30-11-04, due to technical problems with the HPLC equipment.

Table 2 Concentrations of FR-1435X in test medium (static range-finding test).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Nominal [mg/l]	Analysed [mg/l]	Relative to nominal [%]	Relative to initial [%]
0	22-11-04 [1]	30-11-04 [2]	0.05	0.0408	82	
			0.05	0.0431	86	
			0.5	0.394	79	
			0.5	0.396	79	
			5	3.18	64	
			5	4.12	82	
			5	3.79	76	
24	23-11-04 [1]	30-11-04 [2]	0.05	0.0203	41	48
			0.05	0.0193	39	46
			0.5	0.427	85	108
			0.5	0.454	91	115
			5	2.07	41	56
			5	2.26	45	61
			5	2.26	45	61
96	26-11-04 [1]	30-11-04 [2]	0.05	n.d.	n.a.	n.a.
			0.05	n.d.	n.a.	n.a.
			0.5	0.201	40	51
			0.5	0.178	36	45
			5	1.03	21	28
			5	0.978	20	26
			5	0.972	19	26

[1] Samples were frozen until pretreatment.

[2] Test samples were treated on 29-11-04 and stored at ambient temperature until analysis on 30-11-04, due to technical problems with the HPLC equipment.

Table 3 Concentrations of FR-1435X in test medium (flow-through final test).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Target [mg/l]	Analysed [mg/l]	Relative to target [%]	Relative to initial [%]
-24	02-03-05	02-03-05	0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.20	0.160	80	
			0.20	0.163	81	
			0.50	0.413	83	
			0.50	0.409	82	
			1.1	1.01	92	
			1.1	1.01	91	
			1.1	1.02	92	
			2.4	2.43	101	
			2.4	2.41	101	
			2.4	2.42	101	
			5.0	4.49	90	
			5.0	4.49	90	
			5.0	4.47	89	
0	03-03-05	03-03-05	0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.20	0.157	78	
			0.20	0.163	81	
			0.50	0.341	68	
			0.50	0.343	69	
			1.1	0.956	87	
			1.1	0.949	86	
			1.1	0.962	87	
			2.4	2.27	95	
			2.4	2.27	95	
			2.4	2.28	95	
			5.0	4.52	90	
			5.0	4.48	90	
			5.0	4.55	91	
48	05-03-05 [1]	07-03-05	0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.20	0.168	84	105
			0.20	0.169	84	105
			0.50	0.518	104	151
			0.50	0.525	105	153
			1.1	1.06	96	111
			1.1	1.07	97	112
			1.1	1.09	99	114
			2.4	2.17	91	96
			2.4	2.18	91	96
			2.4	2.10	88	92
			5.0	3.62	72	80
			5.0	3.67	73	81
			5.0	3.51	70	78

Table 3 Concentrations of FR-1435X in test medium (flow-through final test) continued.

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Target [mg/l]	Analysed [mg/l]	Relative to target [%]	Relative to initial [%]
72	06-03-05 [1]	07-03-05	5.0	3.63	73	80
			5.0	3.65	73	81
			5.0	3.69 [3]	74	82
92	07-03-05	07-03-05	2.4	2.46	103	108
			2.4	2.47	103	109
			2.4	2.48	103	109
96	07-03-05	07-03-05	0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.20	0.172	86	108
			0.20	0.172	86	107
			0.50	0.487	97	143
			0.50	0.490	98	143
			1.1	0.977	89	102
			1.1	1.01	92	106
			1.1	0.987	90	103

[1] Samples were treated on the day of sampling and stored at ambient temperature until analysis.

[2] Solvent control.

[3] An extra peak was observed in the chromatogram of this sample, this peak was not test substance related.

n.d. Not detected.

n.a. Not applicable.

FIGURES

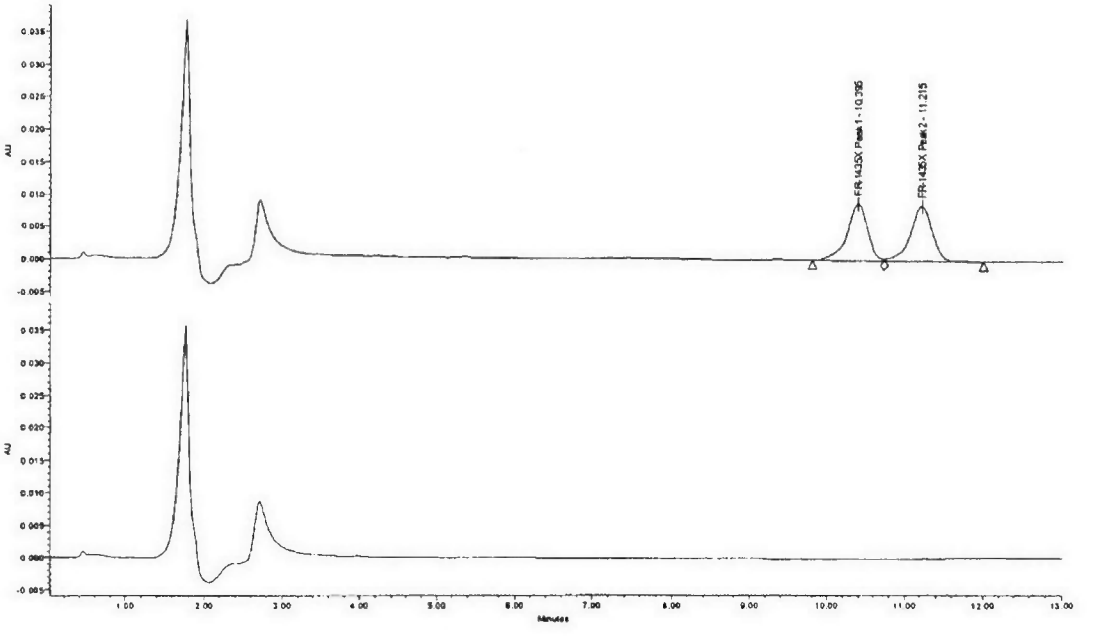


Figure 1 **HPLC chromatograms of a 0.805 mg/l calibration solution (top) and blank 50/50 (v/v) methanol/tap water (bottom) [res.id. 2371 and 2386].**

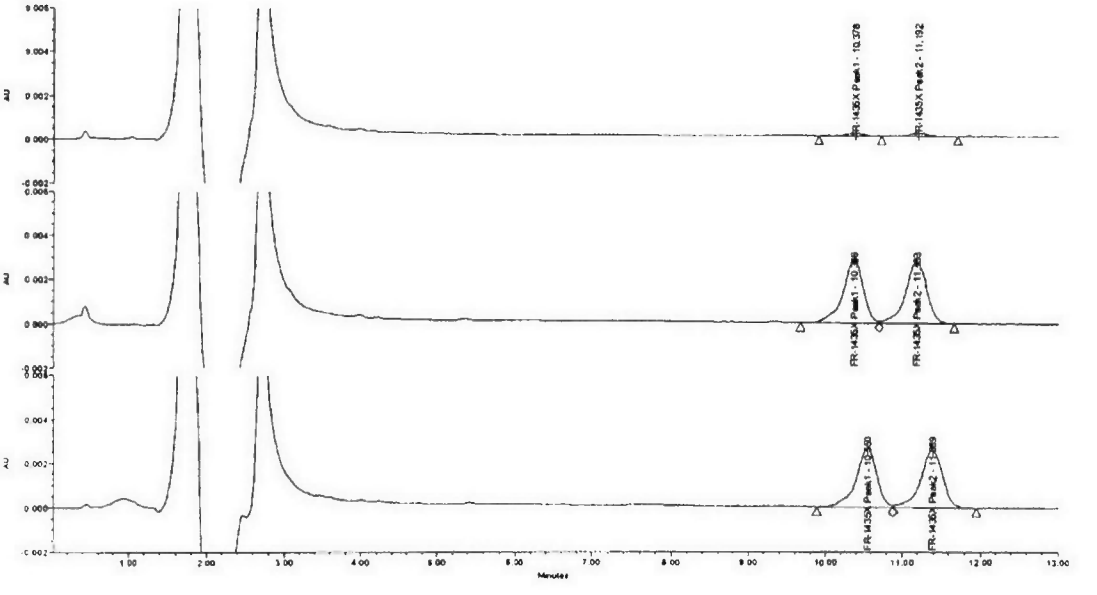


Figure 2 **HPLC chromatograms of procedural recovery samples at concentrations of 0.0251 mg/l [top; res.id. 2629], 0.503 mg/l [middle; res.id. 2669] and 5.03 mg/l [bottom; res.id. 2659].**

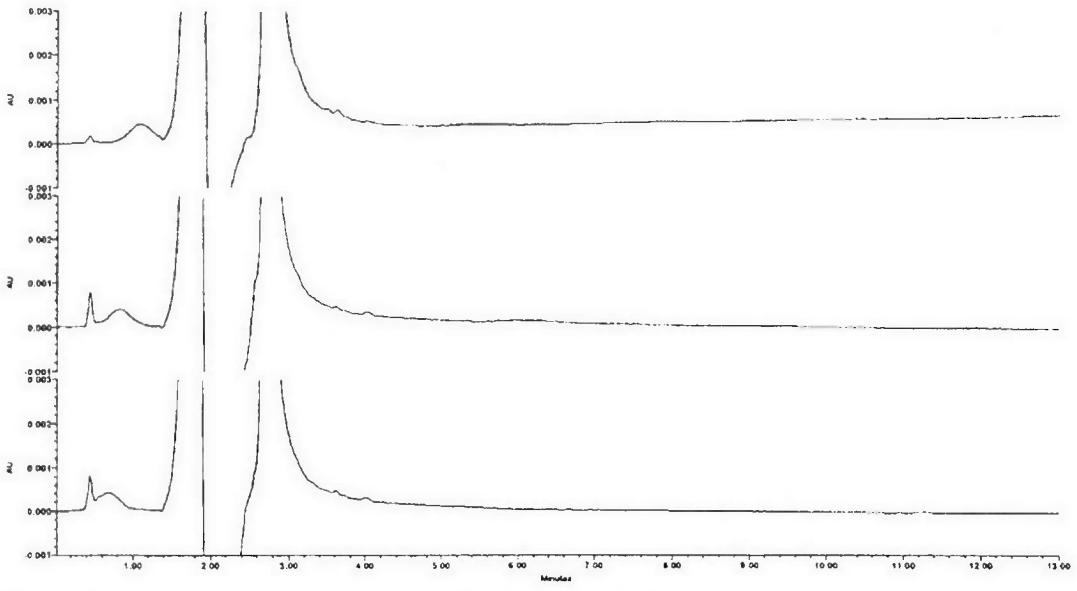


Figure 3 **HPLC chromatograms of solvent-control samples taken at t=0 hours [top; res.id. 2702], at t=48 hours [middle; res.id. 3038] and at t=96 hours [bottom; res.id. 3051].**

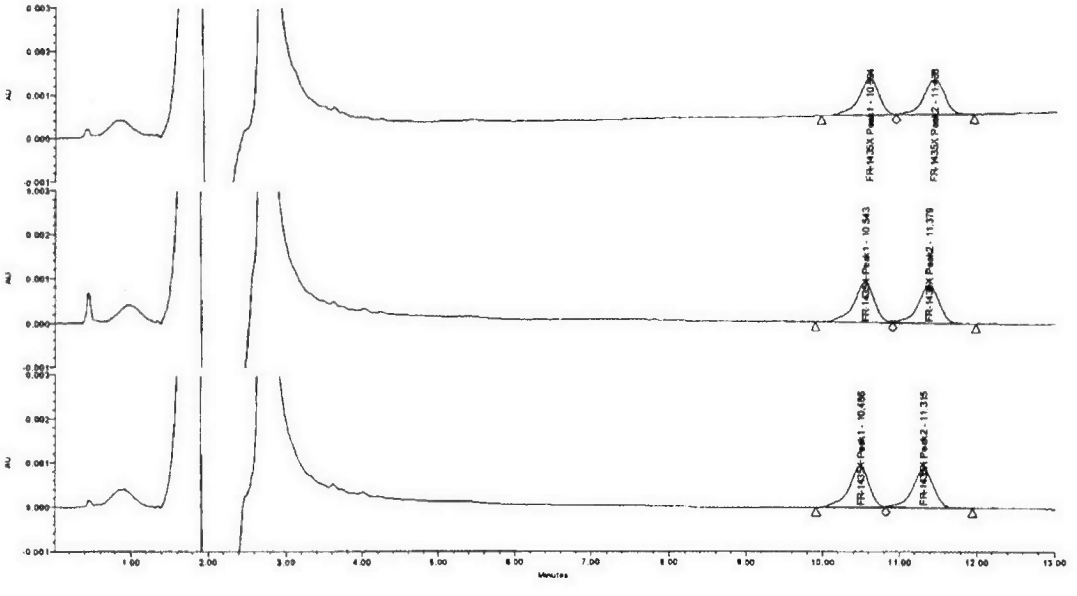


Figure 4 **HPLC chromatograms of target 0.20 mg/l samples taken at t=0 hours [top; res.id. 2703], at t=48 hours [middle; res.id. 3040] and at t=96 hours [bottom; res.id. 3053].**

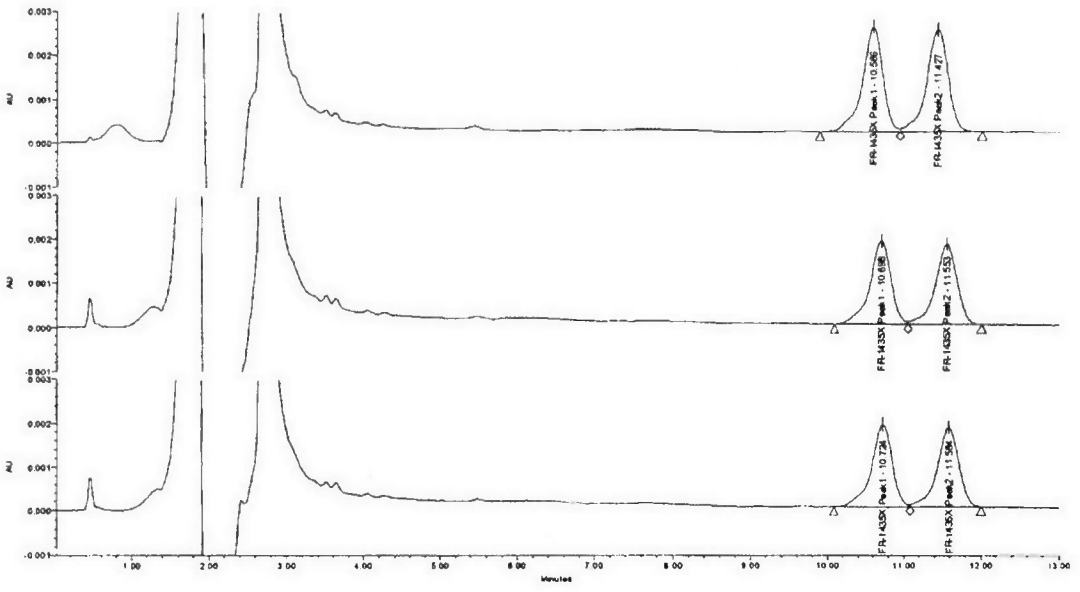


Figure 5 **HPLC chromatograms of target 5.0 mg/l samples taken at t=0 hours [top; res.id. 2721], at t=48 hours [middle; res.id. 3077] and at t=72 hours [bottom; res.id. 3080].**

APPENDIX VI COPY OF THE PROTOCOL + AMENDMENT

PROTOCOL

Study Title

96-HOUR ACUTE TOXICITY STUDY IN CARP WITH FR-1435X (STATIC)

Author

Ir. L.M. Bouwman

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419805
NOTOX Substance 146232/A

1. CONTENTS

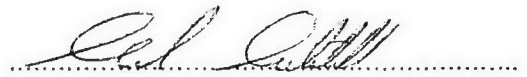
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Ir. L.M. Bouwman

.....

date: 13 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



date: 14-10-04

SPONSOR: Dead Sea Bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

date: 19.10.04

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	Ing. S. Jilesen
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start week beginning : 08 November 2004 (week 46) Completed week beginning : 13 December 2004 (week 51) Proposed Reporting date : 06 February 2005

3.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate acute toxic effects in *Cyprinus carpio* during an exposure period of 96 hours and, if possible, to determine the LC_{50} at all observation times.

3.3. Guidelines

The study procedure described in this protocol is based on the ISO International Standard 7346-1: "Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]" – Part 1: Static method, Second edition, 1996-06-15.

In addition, the procedure is designed to meet the test methods and validity criteria prescribed by the following guidelines and guidance documents:

- European Community (EC), Commission directive 2004/73/EC, Part C: Methods for the determination of ecotoxicity, EC Publication No. L152, April 2004, C.1. "Acute toxicity for fish".
- The OECD guidelines for Testing of Chemicals, guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

This protocol was reviewed and agreed thereafter by the Laboratory Animal Welfare Officer and the Ethical Committee of NOTOX (DEC NOTOX 97-03-06) as required by the Dutch Act on Animal Experimentation (February 1997).

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

3.7. Definitions

Fish are considered to be **dead** when no reaction is observed after touching the caudal peduncle and visible breathing movements are absent.

The **LC_{50}** is the concentration of test substance killing 50% of the fish after a defined period of exposure.

If appropriate, additional definitions may be included in the report (e.g. definitions referring to poorly soluble substances).

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

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Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	
Water	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

The sponsor is responsible for all test substance data unless determined by NOTOX.

4.1.2. Reference substance

The results of a reference test with pentachlorophenol (PCP, SIGMA) will be appended to the report. Due to the stability of the sensitivity of the carps supplied by Zodiac, this reference test is performed once a year.

4.2. Test system

Species	Carp (*Cyprinus carpio*, Teleostei Cyprinidae) Linneaus, 1758.
Source	Depending on availability: Zodiac, proefacc, "De Haar Vissen", L.U. Wageningen, the Netherlands.
Fish length and weight	A representative number (at least 10) of the batch of fish, from which fish will be used for the present study, will be weighed and measured prior to the start of the test.
Mean length of fish	Depending on availability: 2 to 8 cm, with a standard deviation of less than 25 % (EEC: 6 ± 2 cm, OECD: 3 ± 1 cm).

Characteristics	F1 from a single parent-pair bred in UV-treated water. Healthy fish supplied with a health certificate.
Reason for selection	The system has been selected as an internationally accepted species.

4.3. Holding

Quarantine/Acclimatisation	At least 12 days after delivery.
Medium	ISO-medium, formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition: $CaCl_2.2H_2O$ 293.8 mg/l; $MgSO_4.7H_2O$ 123.3 mg/l; $NaHCO_3$ 64.8 mg/l; KCl 5.8 mg/l
Measurements	pH, nitrate and nitrite concentration and ammonia concentration: once a week. Temperature: every day.
Feeding	Daily with Trouvit or Artemia.
Acclimatisation	If fish are not already held in the same medium as the test medium, the fish will be allowed to acclimatise to the test medium without test substance for at least seven days prior to testing after a 48-hour settling-in period.
Validity of batch	In the batch of fish which will be used for the test, mortality during seven days prior to the start of the test has to be less than 5%. If the incidence of mortality is between 5 and 10% acclimatization is continued for seven additional days. If mortality is higher than 10%, the entire batch will be rejected.

4.4. Preparation of stock and test solutions

The exact procedure for preparation of test solutions will be based on a pretest, of which the result will be kept in the raw data.

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable, and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance on the water surface or extensive precipitation, flocculation, aggregation or deposition of the undissolved fraction of the test substance. The method of preparation of the test solutions will be based on data of the test substance supplied by the sponsor and/or on the results of a preliminary test (or specific tests with the test substance performed by NOTOX when the sponsor requests these).

The method of preparation will be based on the principles laid down in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and referred to in the OECD Guidance Document On The Use Of The Harmonised System For The Classification Of Chemicals Which Are Hazardous For The Aquatic Environment, section 3.5: "Difficult to test substances".

The tests will be carried out without adjustment of the pH, except if pH values of the stocks are outside the range of 3-10 and are expected to induce similar extreme pH values in the test solutions.

4.5. Range-finding test

A range-finding test will be performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions will be similar to those applied in the final test with the following exceptions:
Three fish per concentration will be exposed to a range of 0.1 to 100 mg/l increasing by a factor of 10. If applicable a range will be tested up to and including the maximum solubility if this is below 100 mg/l. Dissolved oxygen concentrations and pH will at least be measured in the lowest and highest test concentration. Maximum loading of fish will be 0.5 g fish/litre.

During the exposure period the actual test concentrations should be maintained at 80% or more of the initial concentrations. Determination of the stability of the test substance under test conditions will be performed using samples taken at t= 0, 24 and 96 hours during the range-finding test. The decision which samples will be taken for analysis is made at the start of the test in case samples need to be analysed freshly. Standard procedures assume stability of the test substance under deep freeze conditions. If stability is guaranteed, samples will be taken from all concentrations (except control(s)). The decision which samples will be analysed is then taken by the Study Director at the end of the test period based on the biological results. Optionally, sampling at the end of the test period may be limited to the concentration(s) that are chosen for analysis.
If applicable, the frequency of renewal of the test solutions to be used in the final study will be defined according to the results of this analysis (See also additional procedures).

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

If no toxicity is expected, based on the characteristics of the test substance or other specific information, a limit test or alternatively a limit test combined with a range-finding test will be performed.

4.5.1. Limit test

The limit test will consist of a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Test procedure and conditions will be similar to those applied in the final test. Samples for determination of actual exposure concentrations will be taken from the control and the test concentration at the start, after 24 hours and at the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium different methods of preparation may be combined in order to reach the expected water solubility.

4.5.2. Combined limit/range-finding test

In a combined limit/range-finding test, 7 fish per concentration will be exposed to a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Three fish per concentration will be exposed to 0.1, 1.0 and 10 mg/l. Dissolved oxygen concentrations and pH will at least be measured in the control and the highest test concentration. Samples for determination of actual exposure concentrations will be taken from the control and the highest test concentration at the start, after 24 hours and at the end of the test. No further testing will be

required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

4.6. Final test

4.6.1. Test concentrations

Number	At least 5 concentrations in a geometric series with a factor ≤ 2.2, except when the LC_{50} is expected to be greater than the maximum concentration to be tested. In that case a limit test can be performed.
Range	Preferably, the concentration range has to cover at least one concentration causing no mortality, one or more concentrations causing 10 to 90% mortality and one concentration causing 100% mortality with a maximum concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower.
Controls	Test medium without test substance but treated in the same way as the test substance solutions.

4.6.2. Test procedure and conditions

Test duration	96 hours
Test type	Static without interim renewal of test solutions.
Test vessels	Suitable capacity in relation to the loading, all-glass.
Test medium	ISO-medium, aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilized. The hardness will be 250 mg $CaCO_3$ per litre and the pH 7.8 ± 0.2 after aeration. If necessary, the pH is adjusted to this level by adding NaOH or HCl solution.
Number of fish	A minimum of 7 fish per test group.
Loading	1. Maximum 0.5 g fish/litre, if all test concentrations are > 1.0 mg/l 2. Maximum 0.2 g fish/litre, if one or more test concentrations are < 1.0 mg/l.
Illumination	16 hours photoperiod daily. Alternatively, the 16 hours photoperiod may be performed under yellow light or dimmed light conditions, if the test substance is expected to be photosensitive.
Aeration	The test solutions will not be aerated during the exposure period except when the presence of test substance induces oxygen depletion.

Temperature of medium	20-24°C, constant within 2°C.
Oxygen concentration	> 60% of air saturation value.
pH	Between 6.0 and 8.5. Preferably pH is kept at a variation of not more than 0.2 units, however the validity criterion is set at a maximum variation of 1 unit.
Feeding	No feeding from 48 hours prior to the test and during the total test period.
Introduction of fish	As soon as possible after preparation of the test solutions, fish will be introduced into the test medium, provided that the temperature of the medium is within the optimal range.

4.6.3. Sampling for analysis of test concentrations

Frequency	At the start and the end of the test. This scheme may however be enlarged to include sampling after 24 hours in case the test substance is expected not to be stable in test medium, and a semi-static or flow-through system is not considered appropriate by the Study Director.
Concentrations (standard[1])	Samples for analysis will be taken from three concentrations, i.e. the lowest, a middle and the highest, and the control. Care will be taken not to include any floating layer, test substance film or undissolved material. If all fish should die at the highest concentration before the end of the exposure period, a sample will be taken before removing of the dead fish. At the end of the test a sample will be taken from the highest remaining concentration.
Number of samples	Sampling will consist of singular samples per treatment. Should the analytical validation require duplicate or multiple samples per treatment this will be followed without prior notification.
Volume	Standardly, volumes of 700 µl will be taken, but depending on the limit of detection of the analytical method used in relation to the test concentrations the volume may differ.
Storage	If stability of test concentrations under deep-freeze conditions is ensured, the samples will be stored in a deep-freezer until analysis. Optionally, samples can be stored under different conditions (e.g. room temp.

[1] The standard frequency of sampling is only applicable provided that test solutions are diluted using **one** stock and test concentrations should be **above** 1 mg/l. Sampling will include **all** test solutions if the previous mentioned conditions are not met or if the Study Director decides this is essential for other reasons. Alternatively, sampling may be limited to those test solutions that are biologically relevant.

	or in refrigerator) if stability under these conditions is ensured.
Extra samples	In case singular samples are taken, which are known to be stable under the storage conditions, extra samples will be taken and stored for possible analysis until delivery of the final report with a maximum of three months.
Analyses	When used for analysis the entire volume of each sample will be taken for further dilution or pre-treatment. The analytical method used will be based on the results of a separate project for the development and validation of the analytical method. If study specific adjustments of the analytical method or sample pre-treatment procedures are necessary, these will be developed and tested before the performance of the final test. Detailed specification of these additional analytical procedures will be put down in a protocol amendment (see also 'Additional procedures').

4.6.4. Measurements and recording

Mortality and other effects	At 2-4, 24, 48, 72 and 96 hours following the start of exposure, and every morning from day 1 to observe for any dead fish. Dead fish are removed when observed. Fish that are convulsing or showing other severe forms of distress not considered transient in nature and likely to become more severe before the exposure is terminated, will be sacrificed for humane reasons. These fish will be treated as having died in the test, based on OECD guideline on humane endpoint (ENV/JM/MONO/(2000)7).
Euthanasia	At the end of the test the surviving fish will be rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.
Dissolved oxygen content, pH and temperature of medium	Prior to the introduction of the fish and daily thereafter in all vessels.

4.7. Interpretation

4.7.1. Acceptability of the test

The validity of the test results is based on the following criteria:

1. The proportion of control fish showing abnormal behaviour (including mortality) will not exceed 10% (or one fish if less then ten are used) by the end of the test.
2. The oxygen concentration will be maintained at at least 60% of the air saturation value throughout the test (> 5 mg/l at 22 °C). Other test conditions (pH and temperature) will be maintained within the limits prescribed by this protocol.
3. An analytical program will be included to provide for evidence that the actual test concentrations have been maintained between 80 and 120% of the initial concentrations.
4. The 96h-LC_{50} of the reference chemical if available, for the stock of fish was in reasonable agreement with results obtained previously in the same laboratory.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

4.7.2. Additional procedures

In order to meet the third criterion for the acceptability of the test, additional or alternative procedures will be required for the testing of:

1. Volatile substances;
2. Very toxic or low soluble substances (test concentrations < 1 mg/l);
3. Hydrolytically unstable substances;
4. pH affecting substances;
5. Substances that are not stable under deep-freeze conditions.

These additional procedures may require the amending of:

1. Preparation of test solutions;
2. Additional testing for determination of stability of exposure concentrations;
3. Procedures for maintenance of exposure concentrations (semi-static or flow-through system);
4. The frequency of sampling and analysis for the determination of actual test concentrations;
5. Extension of the analytical program with respect to sample treatment and the sensitivity of the analytical method;
6. If applicable, additional testing with pH adjustment.

The additional procedures are no part of the standard test procedures and will be applied only after emission of an authorised protocol amendment. In such a case the amended procedures will be effective only after NOTOX has received any kind of authorisation from the study monitor.

4.7.3. Data handling

Defining exposure concentrations

1. The results will be based on the nominal or initial (if not in agreement with nominal) test substance concentrations if the analytical program has confirmed that the measured test substance concentrations remained within 20% of the nominal or initial concentrations.
2. If the deviation of the exposure concentrations of the test substance is greater than ± 20% of the nominal or initial concentrations, the results will be expressed in terms of average exposure concentrations. Where measured data are available for the start and end of the test, these concentrations are geometric means calculated from the concentrations measured at the start and end of the test. In case that additional analysis is performed after 24 hours, a time weighed average concentration is calculated. Where at the end of the test measured concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit.

The LC_{50} will be determined using

The maximum likelihood estimation method with the probits of the percentages of dead fish as function of the logarithms of the corresponding concentrations (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition) or using the Logit-model (Cox, D.R., 1977: Analysis of binary data, Methuen & Co. Ltd.), both methods including 95% confidence limits. Optional: data will be analysed using the DEBTOX-model for generation of NEC and EC_x-values as described in 'The Analysis of Aquatic Toxicity Data' by S.A.L.M. Kooijman and J.J.M. Bedaux, 1996.

Should there be no concentration between the highest concentration (A) at which 0% mortality has occurred and the lowest concentration (B) at which 100% mortality has occurred, the LC_{50} will be calculated as $(AB)^{1/2}$, where A and B are limits of the 95% confidence interval.

No LC_{50} can be calculated if the test substance proves to be non-toxic (LC_{50} > maximum concentration).

Optionally, other statistical analyses may be performed if appropriate.

5. DISTRIBUTION

Original:	Study Director
1 Copy:	Technical Coordinator
1 Copy :	Analytical Chemistry
1 Copy:	QAU/Management
1 Copy:	Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO : 1

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

1. Page 6, paragraph 4.1.1. Test substance information:

Description	Brown liquid

REASONS FOR AMENDMENT

1-2 The data has been updated according to information of the sponsor, dated January 06, 2005.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

12 January 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 2

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

1. The final test will be performed applying a flow-through system with continuous renewal of test solutions with the following test conditions:
   - Target concentrations: 0.20, 0.50, 1.1, 2.4 and 5.0 mg/l.
   - Test medium: Tap-water (Hardness 200-220 mg $CaCO_3$/l).
   - Stock solution(s) will be prepared in acetone and dosed via a computer-controlled system connected to a dispenser (Gilson). Via this system the dosed volume enters a mixing flask separately from the tap water supply. The tap water will be supplied via a flow meter. The flow rate will be between 6 and 12 l/h. In the mixing flask the dosed volume and the tap water will be mixed under continuous stirring. The whole system will be checked daily.
   - Fish test vessel: 40 litres (50x30x25 cm) consisting of glass plates sealed with a thin film of silicone and covered by a removable glass or Perspex plate.
   - Before introduction of the fish, test concentrations will be allowed to stabilise for at least 12 hours after the start of the dosing.
   - Samples for analysis will be taken one day before the start of exposure (check functioning system), at the start, after 48 and after 96 hours from the control and all target concentrations.
2. The final test will be performed in week 9. The proposed reporting date will be 10 April 2005.

REASONS FOR AMENDMENT

1 and 2: Analytical results obtained during the range-finding test with carp showed that measured concentrations could not be maintained stable during the test period. During the first 24-hour test period a decrease of more than 20% was observed at nominally 0.05 and 5.0 mg/l. Consequently, test procedures need to be changed to flow-through with continuous renewal to maintain stable concentrations.

PROTOCOL AMENDMENT NO: 2 (continued)

APPROVAL
Study director

Ir. L.M. Bouwman

01 February 2005
date:

Sponsor

Sarit Lifshitz
Dr. S. Lifshitz

6/2/05
date:

PROTOCOL AMENDMENT NO : 3

ORIGINAL

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

During the final flow-through test duplicate or triplicate samples will be taken from the control and all target concentrations.

REASONS FOR AMENDMENT

Duplicate or triplicate samples are required for the analytical validation.

APPROVAL

Study director

.....
Ir. L.M. Bouwman

03 February 2005
.....
date:

Sponsor

Sarit Lifshitz
.....
Dr. S. Lifshitz

6/2/05
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 4

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

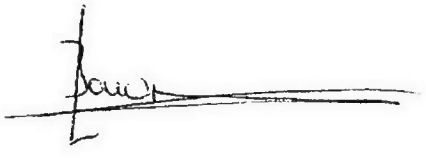
1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director



.....
Ir. L.M. Bouwman

10 February 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 5

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

1. The EEC guideline should read:
- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.1. "Acute toxicity for fish".

REASONS FOR AMENDMENT

1. Correction of the text in the protocol.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

07 April 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 6

Study Title	96-hour acute toxicity study in carp with FR-1435X (static)
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419805

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1. Test substance information:

Density	~2000 kg/m^3

REASONS FOR AMENDMENT

1. The data has been added according to information of the sponsor, dated 15 August 2005 This has no effect on other data.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

26 August 2005
.....
date:

- Attachment 17 -

REPORT

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH FR-1435X

Author

Ir. L.M. Bouwman

Study completion date

20 September 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419827
NOTOX Substance 146232/A

1. CONTENTS

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: 20 September 2005

Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology

i.a. Dr. Ilona C. Enninga
Technical Director

Date: 22/09/2005

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	protocol	14-Oct-04	14-Oct-04	14-Oct-04
	Amendment 1 of protocol	12-Jan-05	12-Jan-05	12-Jan-05
	Amendment 2 of protocol	02-Feb-05	02-Feb-05	02-Feb-05
	Amendment 3 of protocol	04-Feb-05	04-Feb-05	04-Feb-05
	Amendment 4 of protocol	11-Feb-05	11-Feb-05	11-Feb-05
	Amendment 5 of protocol	23-Feb-05	23-Feb-05	23-Feb-05
	Amendment 6 of protocol	08-Apr-05	08-Apr-05	08-Apr-05
	Report	12-Apr-05	12-Apr-05	12-Apr-05
	Amendment 7 of protocol	26-Aug-05	26-Aug-05	26-Aug-05
Process	**Ecotoxicology & Biodegradation** Test substance handling Exposure Observations/Measurements	17-Jan-05	21-Jan-05	21-Jan-05
	Analytical and physical chemistry Test substance handling Observations/Measurements	10-Jan-05	14-Jan-05	20-Jan-05

Head of Quality Assurance
C.J.Mitchell B.Sc.

Date: 26/9/05

4. SUMMARY

Selenastrum capricornutum, Fresh Water Algal Growth Inhibition Test with FR-1435X.

The study procedures described in this report were based on the ISO International Standard 8692, 1989. In addition, the procedures were designed to meet the test methods and validity criteria of the EEC directive 92/69, Part C.3, 1992, the OECD guideline No. 201, 1984 and the OECD series on testing and assessment number 23, 2000.

The batch of FR-1435X tested was a brown liquid with a purity of 97.2%. The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor).

Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone (99.8%, Labscan, Ireland) at concentrations a factor of 10,000 above the target concentrations in test medium. Subsequently, amounts of 50 µl were added to 500 ml M2 medium to obtain the final test concentrations ranging from 0.05 to 5.0 mg/l. After a magnetic stirring period of 15-18 minutes the resulting solutions were clear and colourless, except for the 5.0 mg/l concentration that was slightly hazy.

The study started with a range-finding test exposing exponentially growing algal cultures to nominal FR-1435X concentrations of 0.05, 0.50 and 5.0 mg/l. Both a blank-control group and a solvent-control group were included. The results showed that the expected EC_{50} for cell growth inhibition approximated a nominal concentration of 5.0 mg/l and the expected EC_{50} for growth rate reduction was above a nominal concentration of 5.0 mg/l. Samples taken during the test showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.29 and 2.2 mg/l, respectively. The measured individual concentrations decreased by more than 20% during the test period (46-70% of initial).

The study was continued with a final test exposing exponentially growing algal cultures to nominal FR-1435X concentrations of 0.05, 0.16, 0.51, 1.6 and 5.0 mg/l in silanised glassware. Both a blank-control group and a solvent-control group were included. The initial cell density was $1x10^4$ cells/ml and the total test period was 72 hours. Samples for analytical confirmation of exposure concentrations were taken at the start, after 24 hours of exposure and at the end of the test period.

Analysis of the samples taken at the start of the final test showed that the actual concentrations were in agreement with nominal (81-95%), except for the highest test concentration, which showed a mean initial concentration of 2.9 mg/l (58% of nominal). During the first 24 hours of the test period all actual test concentrations decreased by more than 20% of initial. These results indicated that silanisation of glassware did not prevent the test concentration from decreasing and therefore, that the test substance did not bind to the glassware. The concentrations measured after 24 hours of exposure remained relatively stable during the last 48 hours of exposure. Based on these results, the EC_{50} and the NOEC were based on average exposure concentrations: 0.02, 0.04, 0.12, 0.47 and 0.82 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

FR-1435X inhibited cell growth and reduced growth rate of *Selenastrum capricornutum* significantly at average exposure concentrations of 0.12 mg/l and higher.

The EC_{50} for cell growth inhibition (E_BC_{50}: 0-72h) was 0.56 mg/l with a 95 % confidence interval ranging from 0.15 to 2.1 mg/l. This EC_{50}-value was therefore just above the water solubility of FR-1435X.

As growth rate is derived from the slope under the growth curve in a logarithmic plot, the measure of the specific growth rate is preferable over biomass following from the mathematical nature of exponential growth.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was beyond the range tested and therefore above the water solubility of FR-1435X.

The NOEC for both cell growth inhibition and growth rate reduction was 0.04 mg/l.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	W.J. Koolen
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start : 22 November 2004 Completion : 25 February 2005

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate toxic effects in *Selenastrum capricornutum* during an exposure period of 72 hours and, if possible, to determine the EC_{50} for both inhibition of cell growth and reduction of growth rate for the 72-hour period.

5.3. Guidelines

The study procedures described in this report were based on the ISO International Standard 8692: "Water quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*", First edition, 15 November 1989.

In addition, the procedures were designed to meet the test methods and validity criteria prescribed by the following guidelines and guidance documents:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C-3: "Algal Inhibition Test".
- Organization for Economic Co-operation and Development (OECD), OECD guideline for Testing of Chemicals, guideline No. 201: "Algae, Growth Inhibition Test", Adopted June 7, 1984.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Cell density is the number of cells per millilitre.

Total growth or biomass is defined as the increase in total cell density over the test period. It is derived from the area under the growth curve in a linear plot. The E_BC_{50} is the concentration of test substance that results in a 50% inhibition of total cell growth relative to the control.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Test substance

Identification	FR-1435X
Description	Brown liquid
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000 kg/m^3
Stability in water	Not indicated

6.1.2. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (Appendix V).

6.1.3. Preliminary data

The water solubility of FR-1435X at 20°C is 0.47 mg/l (Material Safety Data Sheet, supplied by the sponsor). FR-1435X concentrations in the range of 0.0254 and 5.08 mg/l in ISO-medium showed to be stable during freezing and thawing of samples (NOTOX Project 419838).

6.2. Test System

Species	*Selenastrum capricornutum*, strain: NIVA CHL 1.
Source	In-house laboratory culture.
Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

6.3. Fresh water algae culture

Stock culture	Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light in a climate room at a temperature of 23 ± 2°C.
Light intensity	60 to 120 $\mu E/m^2/s$ when measured in the photosynthetically effective wavelength range of 400 to 700 nm.
Stock culture medium	M1; formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$NaNO_3$	500	mg/l
K_2HPO_4	40	mg/l
$MgSO_4.7H_2O$	76	mg/l
$Na_2CO_3.10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2.H_2O$	36	mg/l
$C_6H_5FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1.81	mg/l
$ZnCl_2$	0.11	mg/l
$CuSO_4.5H_2O$	0.08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture	3 days (range-finding test) or 4 days (final test) before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of 2.10^4 cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use.

Pre-culture medium

M2; according to the ISO-Standard "Algal growth inhibition test" Nov. 1989; formulated using Milli-Q water (tap water purified by reverse osmosis (milli-RO) and subsequently passed over activated carbon and ion-exchange cartridges: Milli-Q water; Millipore Corp., Bedford, Mass., USA) preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2.2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	80	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l
$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2.6H_2O$	1.5	µg/l
$CuCl_2.2H_2O$	0.01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8.3 ± 0.2	

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system was prevented as much as possible (e.g. film of the test substance on the water surface).

The batch of FR-1435X tested was a brown liquid with a purity of 97.2%.

Due to the low water solubility of the test substance, preparation of test solutions started with stock solutions in acetone (99.8%, Labscan, Ireland) at concentrations a factor of 10,000 above the target concentrations in test medium. Subsequently, amounts of 50 µl were added to 500 ml M2 medium to obtain the final test concentrations ranging from 0.05 to 5.0 mg/l. After a magnetic stirring period of 15-18 minutes the resulting solutions were clear and colourless, except for the 5.0 mg/l concentration that was slightly hazy.

After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, 1 ml of an algal suspension was added to each replicate providing a cell density of 10^4 cells/ml.

In the final test, all glassware was silanised prior to use. To this end, glassware was rinsed three times with 2% dichlorodimethylsilane in heptane. Subsequently, heptane was evaporated under pressure air. Finally, glassware was rinsed three times with purified water and dried in an incubator at 85°C.

6.5. Range-finding test

A range-finding test was performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those applied in the final test with the following exceptions: Exponentially growing algal cultures were exposed to 0.05, 0.50 and 5.0 mg/l and to two control groups (i.e. a solvent-control and a blank-control). Three replicates were tested per concentration and three replicates in the control groups. pH was only measured in the control groups and the highest test concentration. Samples for possible analysis were taken from all concentrations at the start and the end of the test. These samples were stored in a freezer until analysis.

6.6. Final test

6.6.1. Test concentrations

FR-1435X	0.05, 0.16, 0.51, 1.6 and 5.0 mg/l
Controls	Test medium without test substance or other additives (blank-control) and one control containing acetone as used in the treatment of the stock solutions (solvent-control).
Replicates	3 replicates of each test concentration 6 replicates of both control groups 2 replicates of the highest test solution without algae 1 extra replicate of each test solution for sampling purposes

6.6.2. Test procedures and conditions

Test duration	72 hours
Test type	Static
Test vessels	100 ml, all-glass, containing 50 ml of test solution
Medium	M2
Cell density	An initial cell density of 1 x 10^4 cells/ml.
Illumination	Continuously using TLD-lamps of the type 'Cool-white' of 30 Watt, with a light intensity within the range of 68 to 95 $\mu E.m^{-2}.s^{-1}$.
Incubation	Vessels were distributed at random in the incubator. During incubation the algal cells were kept in suspension by continuous shaking.

6.6.3. Sampling for analysis of test concentrations

During the final test duplicate samples were taken from concentrations < 1.0 mg/l and triplicate samples were taken from concentrations ≥ 1.0 mg/l. The method of analysis is described in the appended Analytical Report (Appendix VII).

Frequency	at t=0 h, t=24 h and t=72 h
Volume	700 μl
Storage	Samples taken at t=0 h and t=24 h were stored in a freezer until analysis. Samples taken at t=72 h were analysed on the day of sampling.

Compliance with the Quality criteria regarding maintenance of actual concentrations was demonstrated by running a test vessel at the highest test concentration but without algae and samples for analysis were taken at the start, after 24 hours of exposure and at the end of the test period.

6.6.4. Measurements

pH	At the beginning and at the end of the test. The pH of the solutions should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel

6.6.5. Recording of cell densities

At the beginning of the test, cells were counted using a microscope and a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Varian Cary 50 single beam spectrophotometer with immersion probe (pathlength =20 mm). Varian Nederland BV., Houten, The Netherlands. Algal medium was used as blank.

6.7. Interpretation

6.7.1. Data handling

Calibration curve
Quantification of cell densities was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of areas under the growth curves
The area below the growth curve was calculated using the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2 \times N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2 \times N_0}{2} \times (t_n - t_{n-1})$$

Where: A = area
N_0= nominal number of cells/ml at the start of the test
N_1= measured number of cells/ml at t_1
N_n= measured number of cells/ml at t_n
t_1= time of first measurements after beginning of the test
t_n= time of n^{th} measurement after beginning of the test

The percentage inhibition of cell growth at each test concentration (I_T) was calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} \times 100$$

Where: A_C = area below the growth curve obtained in the pooled controls
A_T = area below the growth curve at each test substance concentration

Growth inhibition was calculated for the total period of 72h.

Comparison of growth rates
The average specific growth rate (μ) for exponentially growing cultures was calculated as:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

The average growth rate at each test substance concentration was then compared to the control value and the percentage reduction in growth rate was calculated.

Determination of the average exposure concentrations
The average exposure concentrations were calculated as:
$\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 48 \times \sqrt{C_{t=24} \times C_{t=72}}}{72}$, being the Time Weighed Average of the concentrations of FR-1435X measured in the samples taken at the start ($C_{t=0}$), after 24 hours ($C_{t=24}$) and the end of the test ($C_{t=72}$).

Determination of the NOEC and calculation of the EC_{50}
For determination of the NOEC and the EC_{50} the approaches recommended in the OECD guideline (201, adopted 7 June 1984) were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant reduction of growth or inhibition of growth rate (Two sample t test for the controls, ANOVA, Bonferroni t test, Tukey test, TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Additionally, the EC_{10} was determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993. Calculation of the EC_{50} and EC_{10} values was based on log-linear regression analysis of the percentages of growth inhibition and the percentages of growth rate reduction versus the logarithms of the corresponding initial exposure concentrations of the test substance.

6.7.2. Acceptability of the test

1. In the controls, cell density increased by an average factor of > 16 within 3 days.
2. Further, test conditions (temperature and pH) remained within the ranges prescribed by the guideline.

6.8. List of protocol deviations

There were no deviations from the protocol.

7. RESULTS

7.1. Range-finding test

7.1.1. Mean cell densities, inhibition of cell growth and reduction of growth rate

The mean cell densities measured during the range-finding test are presented in Table 1. Table 2 presents the percentages growth inhibition and growth rate reduction per concentration. The results showed that the expected EC_{50} for cell growth inhibition approximated a nominal concentration of 5.0 mg/l, while the expected EC_{50} for growth rate reduction was above a nominal concentration of 5.0 mg/l.

All test conditions were maintained within the limits prescribed by the protocol, except for the pH of the control groups that increased above 9.0 during the test period. This could be related to a relatively high rate of algal growth.

Table 1 **Mean cell densities ($x10^4$ cells/ml) during the range-finding test**

Nominal conc. FR-1435X (mg/l)	Exposure time (hours)			
	0	24	48	72
Blank-control	1.0	2.4	16.6	73.9
Solvent-control	1.0	3.6	18.8	78.4
0.05	1.0	3.6	17.9	78.7
0.5	1.0	3.6	14.3	56.6
5.0	1.0	2.9	9.3	33.2

Table 2 **Percentage reduction of growth rate and inhibition of total growth during the range-finding test**

Nominal conc. FR-1435X (mg/l)	Cell growth (0-72 hrs)		Mean growth rate	
	Mean area (A)	Inhibition (%)	μ (0-72 hrs)	Reduction (%)
Blank-control	1284.32		0.05975	
Solvent-control	1419.56		0.06052	
0.05	1401.16	1.3	0.06062	-0.2
0.5	1048.60	26.1	0.05605	7.4
5.0	632.52	55.4	0.04864	19.6

7.1.2. Stability of FR-1435X under test conditions

The analytical results showed that the mean initial FR-1435X concentrations at nominal 0.05, 0.50 and 5.0 mg/l were 0.04, 0.29 and 2.2 mg/l, respectively. The measured individual concentrations decreased by more than 20% during the test period (46-70% of initial, see Table 2 of the appended Analytical report).

7.2. Final test

7.2.1. Measured test substance concentrations

The results of analysis of the samples taken during the final test are described in Table 3 of the appended Analytical Report.
Analysis of the samples taken at the start (t=0) of the final test showed that the actual concentrations were in agreement with nominal (81-95%), except for the highest test concentration, which showed a mean initial concentration of 2.9 mg/l (58% of nominal). During the first 24 hours of the test period all actual test concentrations, including the concentration in the vessels without algae, decreased by more than 20% of initial. These results indicated that silanisation of glassware did not prevent the test concentration from decreasing and therefore, that the test substance did not bind to the glassware. The major deviation of nominal at the highest test concentration at the start of the test can be explained by the low water solubility of the test substance. The decrease of test substance concentrations (all vessels) during the first 24 hours can be attributed to degradation of the test substance (see also NOTOX Project 419849). the low water solubility of the test substance. The concentrations measured after 24 hours of exposure remained relatively stable during the last 48 hours of exposure. Based on these results, the EC_{50} and the NOEC were based on average exposure concentrations: 0.02, 0.04, 0.12, 0.47 and 0.82 mg/l.

Note that during the last 48 hours of exposure actual test concentrations seem to have increased in the vessels containing average exposure concentrations of 0.04, 0.12 and 0.47 mg/l and in the vessels containing the highest concentration without algae. No explanation can be given for these results. However, it did not affect the validity criteria of the study because cell growth inhibition and growth rate reduction proved to be related to this concentration increase (see Tables 4 and 5).

7.2.2. Mean cell densities

Table 3 shows mean cell densities measured at 24-hour intervals at the different concentrations of FR-1435X. The respective growth curves are shown in Figure 1 (see Appendix I for the cell densities per replicate). Note that variation between replicates was observed at all test concentrations. One replicate of the highest test concentration was considered an outlier, since the algal density was higher in this vessel from the start of the test.

Table 3 Mean cell densities (x 10^4 cells/ml) during the final test

Average conc. FR-1435X (mg/l)	Exposure time (hours)			
	0	24	48	72
Blank-control	1.0	3.0	7.9	32.0
Solvent-control	1.0	2.6	7.8	31.5
0.02	1.0	2.5	7.1	27.8
0.04	1.0	2.9	9.2	32.1
0.12	1.0	2.6	7.2	23.1
0.47	1.0	2.2	5.6	17.0
0.82	1.0	2.3	4.4	9.8

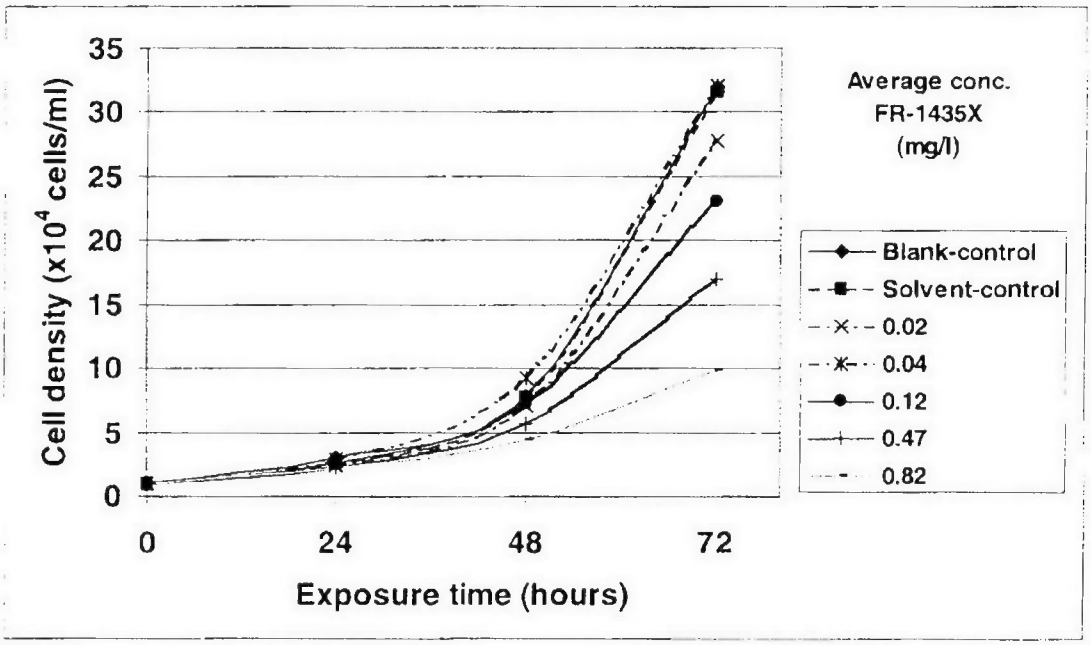

Figure 1 Growth curves at different concentrations of FR-1435X

7.2.3. Inhibition of cell growth and reduction of growth rate

Table 4 shows the calculation of the percentages of inhibition of cell growth and Table 5 the percentages of growth rate reduction at different time intervals (see Appendix I for the areas of cell growth and values of growth rate per replicate). Statistical analysis of the data for areas under the growth curves (cell growth) and growth rate are shown in Appendices II and III. Due to variation between replicates of the test concentrations both control groups were combined for statistical analysis of the data. By combination of the control groups, i.e. using 12 replicates instead of six, the statistical analysis of the test concentrations becomes more accurate. Statistical justification for combination of control groups was tested with a t test (see Appendices II and III).

Inhibition of cell growth increased with increasing concentration of FR-1435X from 0.12 mg/l upwards resulting in 62% inhibition at 0.82 mg/l. Statistically significant inhibition of cell growth was found at test concentrations of 0.12 mg/l and higher (Bonferroni t and Tukey test, $\alpha = 0.05$).

Growth rates were in the range of the controls at concentrations of 0.02 and 0.04 mg/l during the 72-hour test period, whereas the growth rate of algae exposed to 0.12 mg/l and higher were increasingly reduced. Reduction of growth rate had decreased after 48 hours and had increased again after 72 hours. This was related to the actual measured concentrations. Statistically significant reduction of growth rate was found at test concentrations of 0.12 mg/l and higher (Bonferroni t and Tukey test, $\alpha = 0.05$).

Table 4 Percentage inhibition of cell growth during the final test

Average conc. FR-1435X (mg/l)	Cell growth (0-72 hrs)	
	Mean area (A)	Inhibition (%)
Blank-control	584.60	
Solvent-control	566.36	
0.02	503.28	12.5
0.04	615.36	-6.9
0.12	453.32	21.2
0.47	333.44	42.1
0.82	220.50	61.7

Table 5 Percentage reduction of growth rate at different time intervals during the final test

Average conc. FR-1435X (mg/l)	Mean growth rate					
	μ (0-24 hrs)	Reduction (%)	μ (0-48 hrs)	Reduction (%)	μ (0-72 hrs)	Reduction (%)
Blank-control	0.04506		0.04291		0.04810	
Solvent-control	0.03915		0.04265		0.04792	
0.02	0.03705	12.0	0.04075	4.8	0.04609	4.0
0.04	0.04397	-4.4	0.04622	-8.0	0.04818	-0.3
0.12	0.03908	7.2	0.04105	4.1	0.04345	9.5
0.47	0.03297	21.7	0.03580	16.3	0.03898	18.8
0.82	0.03517	16.5	0.03098	27.6	0.03176	33.8

7.2.4. Determination of effect concentrations

Table 6 shows the effect parameters based on average exposure concentrations, see also Appendix IV.

Table 6 Effect parameters

Parameter	Concentration FR-1435X (mg/l)	95%-confidence interval
NOE_BC	0.04	
72h-E_BC_{10}	0.08	0.02 - 0.31
72h-E_BC_{50}	0.56	0.15 - 2.1
NOE_RC	0.04	
72h-E_RC_{10}	0.12	0.03 - 0.53
72h-E_RC_{50}	> 0.82	

7.2.5. Experimental conditions

Table 7 shows the pH recorded at the beginning and the end of the test. The pH was within the limits prescribed by the protocol (6.0-9.0, preferably not varying by more than 1.5 unit). The temperature of the test medium was 21.9°C at the start of the test. During the exposure period the temperature measured in the incubator was maintained between 22.2 and 22.6°C. Temperature remained within the limits prescribed by the protocol (21-25°C, constant within 2°C).

Table 7 pH levels recorded during the final test

Initial conc. FR-1435X (mg/l)	Exposure time (hours)	
	0	72
Blank-control	8.3	8.2
Solvent-control	8.3	8.3
0.08	8.2	8.2
0.18	8.2	8.2
0.33	8.2	8.3
0.78	8.2	8.2
1.5	8.2	8.1

8. CONCLUSION

Under the conditions of the present study with *Selenastrum capricornutum*, FR-1435X inhibited cell growth and reduced growth rate of this fresh water algae species significantly at average exposure concentrations of 0.12 mg/l and higher.

The EC_{50} for cell growth inhibition (E_BC_{50}: 0-72h) was 0.56 mg/l with a 95 % confidence interval ranging from 0.15 to 2.1 mg/l. This EC_{50}-value was therefore just above the water solubility of FR-1435X.

As growth rate is derived from the slope under the growth curve in a logarithmic plot, the measure of the specific growth rate is preferable over biomass following from the mathematical nature of exponential growth.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was beyond the range tested and therefore above the water solubility of FR-1435X.

The NOEC for both cell growth inhibition and growth rate reduction was 0.04 mg/l.

APPENDIX I WORKSHEET DATA

Table 8 Individual cell densities

Number of inoculated cells at t=0: $1x10^4$ cells/ml					
Average conc. FR-1435X (mg/l)	Vessel number	Exposure time (hours)			
		0	24	48	72
Blank-control	1	1.00	2.45	7.87	31.81
	2	1.00	2.71	7.90	31.40
	3	1.00	4.45	7.92	30.15
	4	1.00	2.74	7.18	28.70
	5	1.00	2.84	7.90	34.84
	6	1.00	2.86	8.34	35.08
Solvent-control	1	1.00	2.79	8.35	33.26
	2	1.00	2.73	8.00	31.92
	3	1.00	2.75	8.23	30.98
	4	1.00	2.49	7.77	30.61
	5	1.00	2.13	7.09	30.82
	6	1.00	2.54	7.13	31.59
0.02	1	1.00	2.03	6.23	27.78
	2	1.00	2.97	8.39	31.21
	3	1.00	2.39	6.76	24.29
0.04	1	1.00	2.88	9.23	32.76
	2	1.00	2.70	8.73	30.45
	3	1.00	3.05	9.65	33.15
0.12	1	1.00	2.78	8.42	26.83
	2	1.00	1.91	6.25	18.51
	3	1.00	3.14	7.01	23.97
0.47	1	1.00	2.74	6.69	21.69
	2	1.00	1.93	4.53	12.12
	3	1.00	2.03	5.72	17.27
0.82	1	1.00	2.19	4.12	9.98
	2*	1.00	3.98	7.74	20.26
	3	1.00	2.47	4.75	9.71

* Replicate was considered an outlier and was therefore not included in the calculation of mean cell densities.

APPENDIX I WORKSHEET DATA – continued –

Table 9 Calculation of growth (area under growth curve) and growth rate

Average conc. FR-1435X (mg/l)	Vessel number	Area (A)	Growth rate			Growth inhib. (%)	Growth rate reduction (%)		
		0-72 hrs	0-24 hrs	0-48 hrs	0-72 hrs	0-72 hrs	0-24 hrs	0-48 hrs	0-72 hrs
Blank-control	1	569.40	0.03734	0.04298	0.04805				
	2	571.44	0.04154	0.04306	0.04787				
	3	598.68	0.06220	0.04311	0.04731				
	4	522.48	0.04200	0.04107	0.04662				
	5	615.84	0.04349	0.04306	0.04932				
	6	629.76	0.04378	0.04419	0.04941				
Solvent-control	1	606.48	0.04275	0.04421	0.04867				
	2	580.56	0.04185	0.04332	0.04810				
	3	575.28	0.04215	0.04391	0.04769				
	4	553.56	0.03801	0.04271	0.04752				
	5	531.12	0.03151	0.04081	0.04761				
	6	551.16	0.03884	0.04092	0.04796				
Mean						575	0.04212	0.04278	0.04801
0.02	1	471.60	0.02950	0.03811	0.04617	18	30	11	4
	2	587.16	0.04536	0.04431	0.04779	-2	-8	-4	0
	3	451.08	0.03630	0.03981	0.04431	22	14	7	8
0.04	1	623.76	0.04407	0.04630	0.04846	-8	-5	-8	-1
	2	579.72	0.04139	0.04514	0.04745	-1	2	-6	1
	3	642.60	0.04646	0.04723	0.04863	-12	-10	-10	-1
0.12	1	530.76	0.04260	0.04439	0.04569	8	-1	-4	5
	2	357.96	0.02696	0.03818	0.04053	38	36	11	16
	3	471.24	0.04768	0.04057	0.04412	18	-13	5	8
0.47	1	426.60	0.04200	0.03960	0.04273	26	0	7	11
	2	240.48	0.02740	0.03147	0.03465	58	35	26	28
	3	333.24	0.02950	0.03633	0.03957	42	30	15	18
0.82	1	211.20	0.03266	0.02950	0.03195	63	22	31	33
	2*	464.40	0.05755	0.04263	0.04179	19	-37	0	13
	3	229.80	0.03768	0.03246	0.03157	60	11	24	34

* Replicate was considered an outlier and was therefore not included in the calculation of mean growth and growth rate.

APPENDIX II STATISTICS: CELL GROWTH (0-72 HOURS)

TOXSTAT.EXE

```
      t-Test of Solvent and Blank Controls          Ho: GRP1 Mean = GRP2 Mean
=============================================================================
GRP1 (Solvent cntl) Mean =    584.6000      Calculated t value =      0.9524
GRP2 (Blank cntl) Mean   =    566.3600      Degrees of freedom =     10
Difference in means      =     18.2400
=============================================================================
2-sided t value (0.05,10) = 2.2281  No significant difference at alpha=0.05
2-sided t value (0.01,10) = 3.1693  No significant difference at alpha=0.01

    WARNING:  This procedure assumes normality and equal variances!

                        Print this table? (Y/N) ==> _
```

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.7420	6.2920	9.9320	6.2920	1.7420
OBSERVED	1	8	8	8	1

Chi-Square = 1.9352 (p-value = 0.7477)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Hartley's Test for Homogeneity of Variance

Calculated H statistic (max Var/min Var) = 50.0647

Table H statistic = 184.00 (alpha = 0.01)
62.00 (alpha = 0.05)

Used Closest df = 3
(Actual value: df (avg # reps - 1) = 3.33)
Based on R (# groups) = 6

Data PASS homogeneity test (alpha = 0.01). Continue analysis.

APPENDIX II STATISTICS: CELL GROWTH (0-72 HOURS) – continued –

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	354174.6367	70834.9273	24.5297
Within (Error)	20	57754.4616	2887.7231	
Total	25	411929.0983		

(p-value = 0.0000)

Critical F = 4.1027 (alpha = 0.01, df = 5,20)
= 2.7109 (alpha = 0.05, df = 5,20)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	GRPS 1&2 POOLED	575.4800	575.4800		
2	0.02	503.2800	503.2800	2.0814	
3	0.04	615.3600	615.3600	1.1497	
4	0.12	453.3200	453.3200	3.5217	*
5	0.47	333.4400	333.4400	6.9777	*
6	0.82	220.5000	220.5000	8.6490	*

Bonferroni t critical value = 2.8453 (2 Tailed, alpha = 0.05, df = 5,20)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	GRPS 1&2 POOLED	12			
2	0.02	3	98.6975	17.2	72.2000
3	0.04	3	98.6975	17.2	39.8800
4	0.12	3	98.6975	17.2	122.1600
5	0.47	3	98.6975	17.2	242.0400
6	0.82	2	116.7805	20.3	354.9800

Tukey Method of Multiple Comparisons

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 0 6	0 5	0 4	0 2	0 1	0 3
6	0.82	220.5000	220.5000	\					
5	0.47	333.4400	333.4400	.	\				
4	0.12	453.3200	453.3200	*	.	\			
2	0.02	503.2800	503.2800	*	*	.	\		
1	GRPS 1&2 POOLED	575.4800	575.4800	*	*	*	.	\	
3	0.04	615.3600	615.3600	*	*	*	.	.	\

* = significant difference (alpha = 0.05) . = no significant difference

Tukey critical value = 4.4450 (df = 6,20) s = 2887.7231

APPENDIX III STATISTICS: GROWTH RATE (0-72 HOURS)

TOXSTAT.EXE

```
      t-Test of Solvent and Blank Controls          Ho: GRP1 Mean = GRP2 Mean
=============================================================================
GRP1 (Solvent cntl) Mean =      0.0481     Calculated t value =      0.3559
GRP2 (Blank cntl) Mean   =      0.0479     Degrees of freedom =     10
Difference in means      =      0.0002
=============================================================================
2-sided t value (0.05,10) = 2.2281  No significant difference at alpha=0.05
2-sided t value (0.01,10) = 3.1693  No significant difference at alpha=0.01

    WARNING:  This procedure assumes normality and equal variances!


                        Print this table? (Y/N) ==> _
```

Transform: NATURAL LOG(Y)

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.7420	6.2920	9.9320	6.2920	1.7420
OBSERVED	1	7	10	6	2

Chi-Square = 0.4479 (p-value = 0.9784)

Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Hartley's Test for Homogeneity of Variance

Calculated H statistic (max Var/min Var) = 157.0879

Table H statistic = 184.00 (alpha = 0.01)
62.00 (alpha = 0.05)

Used Closest df = 3
(Actual value: df (avg # reps - 1) = 3.33)
Based on R (# groups) = 6

Data PASS homogeneity test (alpha = 0.01). Continue analysis.

APPENDIX III STATISTICS: GROWTH RATE (0-72 HOURS) – continued –

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.3761	0.0752	41.3058
Within (Error)	20	0.0364	0.0018	
Total	25	0.4126		

(p-value = 0.0000)

Critical F = 4.1027 (alpha = 0.01, df = 5,20)
= 2.7109 (alpha = 0.05, df = 5,20)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Transform: NATURAL LOG(Y)

Bonferroni t-Test - **TABLE 1 OF 2** Ho: Control=Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	TRANS t STAT	SIG 0.05
1	GRPS 1&2 POOLED	-3.0365	0.0480		
2	0.02	-3.0776	0.0461	1.4949	
3	0.04	-3.0329	0.0482	0.1302	
4	0.12	-3.1375	0.0434	3.6672	*
5	0.47	-3.2483	0.0390	7.6915	*
6	0.82	-3.4496	0.0318	12.6744	*

Bonferroni t critical value = 2.8453 (2 Tailed, alpha = 0.05, df = 5,20)

Bonferroni t-Test - **TABLE 2 OF 2** Ho: Control=Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	GRPS 1&2 POOLED	12			
2	0.02	3	0.0039	8.2	0.0019
3	0.04	3	0.0039	8.2	0.0002
4	0.12	3	0.0039	8.2	0.0046
5	0.47	3	0.0039	8.2	0.0090
6	0.82	2	0.0047	9.7	0.0163

Tukey Method of Multiple Comparisons

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 6	5	4	2	1	3
6	0.82	-3.4496	0.0318	\					
5	0.47	-3.2483	0.0390	*	\				
4	0.12	-3.1375	0.0434	*	*	\			
2	0.02	-3.0776	0.0461	*	*	.	\		
1	GRPS 1&2 POOLED	-3.0365	0.0480	*	*	*	.	\	
3	0.04	-3.0329	0.0482	*	*	.	.	.	\

* = significant difference (alpha = 0.05) . = no significant difference
Tukey critical value = 4.4450 (df = 6,20) s = 0.0018

APPENDIX IV EC-VALUES

Table 10 EC-values for growth inhibition

Concentration (mg/l)	X Log conc. (mg/l)	Y Inhibition (%)
0.02	*	18.1
0.02	*	-2.0
0.02	*	21.6
0.04	-1.398	-8.4
0.04	-1.398	-0.7
0.04	-1.398	-11.7
0.12	-0.921	7.8
0.12	-0.921	37.8
0.12	-0.921	18.1
0.47	-0.328	25.9
0.47	-0.328	58.2
0.47	-0.328	42.1
0.82	-0.086	63.3
0.82	*	19.3
0.82	-0.086	60.1

* Not included in the EC calculations

Slope:	48.5624
Intercept:	62.3994
Multiple R:	0.9199
n = number of observations:	11

Regression line: Y= 48.56X + 62.40

Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	0.08	0.02	0.31
25	0.17	0.05	0.61
50	0.56	0.15	2.11

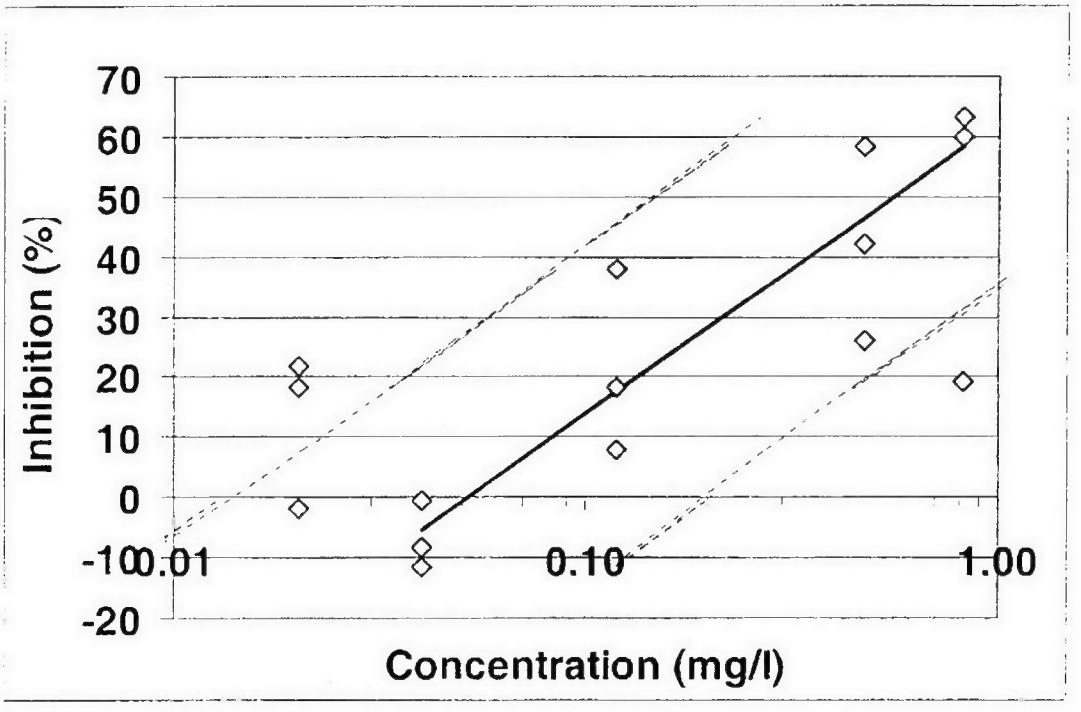

Figure 2 Percentage inhibition of cell growth as function of the log concentration (mg/l) of FR-1435X. Dashed curves represent the 95% confidence limits.

APPENDIX IV EC-VALUES – continued –

Table 11 EC-values for growth rate reduction

Concentration (mg/l)	X Log conc. (mg/l)	Y Reduction (%)
0.02	*	3.8
0.02	*	0.5
0.02	*	7.7
0.04	-1.398	-0.9
0.04	-1.398	1.2
0.04	-1.398	-1.3
0.12	-0.921	4.8
0.12	-0.921	15.6
0.12	-0.921	8.1
0.47	-0.328	11.0
0.47	-0.328	27.8
0.47	-0.328	17.6
0.82	-0.086	33.4
0.82	*	13.0
0.82	-0.086	34.2

* Not included in the EC calculations

Slope:	22.6889
Intercept:	30.5112
Multiple R:	0.9031
n = number of observations:	11

Regression line: Y= 22.69X + 30.51

Prediction of X values based on known Y values

Known Y Reduction (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	0.12	0.03	0.53
25	0.57	0.13	2.54
50	7.23	1.03	50.63

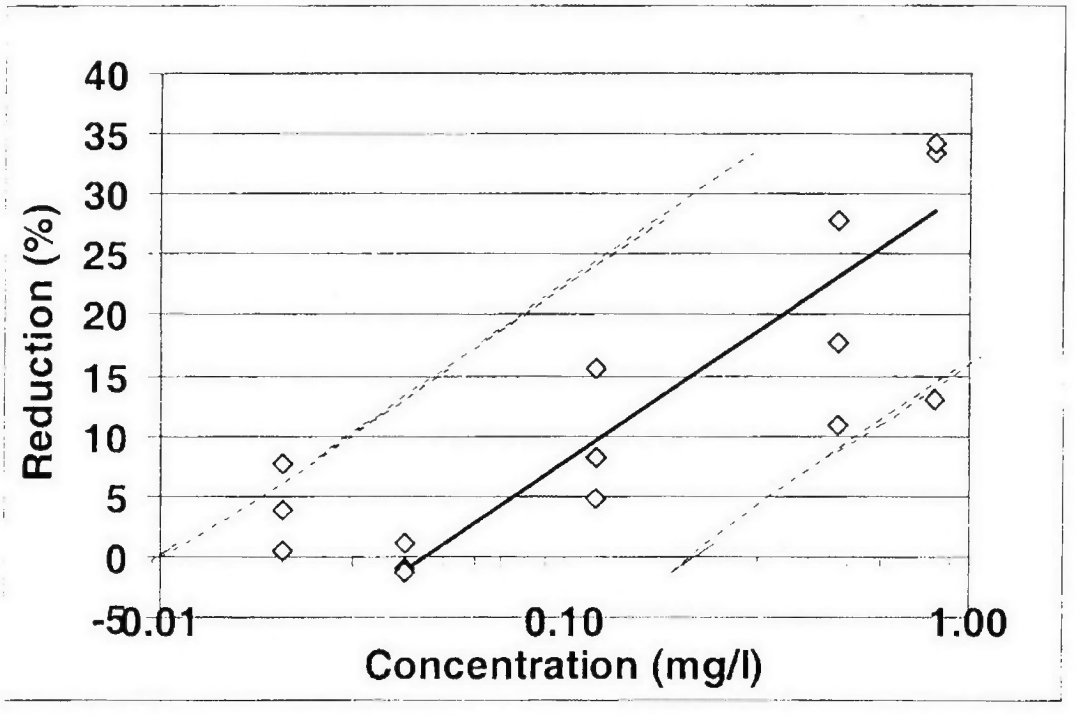

Figure 3 Percentage reduction of growth rate as function of the log concentration (mg/l) of FR-1435X. Dashed curves represent the 95% confidence limits.

APPENDIX V REFERENCE TEST

Selenastrum capricornutum, strain: NIVA CHL 1. Fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 425677).

Start of first exposure: 29 November 2004
Completion last exposure: 02 December 2004

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted June 7, 1984; and ISO Standard 8692, First edition, 15 November 1989.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to Potassium dichromate (Merck, Art. 4864, Batch K28974764).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ (Potassium dichromate) concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a blank-control. The initial cell density was 1.0 x 10^4 cells/ml.

Results:

Overview of % inhibition in cell growth and % reduction of growth rate in the reference test:

Concentration $K_2Cr_2O_7$ (mg/l)	Total cell growth: Mean Area 0-72h	Inhibition %	Growth rate: Interval 0-72h	Reduction %
Blank-control	1790.54		0.06372	
0.18	1713.28	4.3	0.06272	1.6
0.32	1495.00	16.5	0.06093	4.4
0.56	1019.52	43.1	0.05415	15.0
1.0	418.96	76.6	0.03807	40.3
1.8	169.48	90.5	0.01874	70.6
3.2	140.08	92.2	0.01521	76.1

Under the conditions of the reference study with *Selenastrum capricornutum*, potassium dichromate reduced growth rate of this fresh water algae species at nominal concentrations of 0.56 mg/l and higher.

The EC_{50} for cell growth inhibition (E_BC_{50}: 0-72h) was 0.62 mg/l with a 95% confidence interval ranging from 0.41 to 0.94 mg/l. The historical ranges of the 72h EC_{50} for growth inhibition lie between 0.49 and 1.4 mg/l. Hence, the E_BC_{50}: 0-72h for the present batch corresponds with this range.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 1.2 mg/l with a 95% confidence interval ranging from 0.82 to 1.7 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Hence, the E_RC_{50}: 0-72h for the present batch corresponds with this range.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

FR-1435X

NOTOX Project 419827

APPENDIX VI CERTIFICATE OF ANALYSIS

IMI (TAMI) Institute for Research & Development Ltd
Flame Retardants Department
P.O.Box 10140, Haifa Bay 26111, Israel Tel.+972-4-8469553
Fax+972-4-846-9320 E-mail:zilbermanj@tami-imi.co.il

תמי (אימי) מכון למחקר ולפתוח בעיים
מחלקת עכבי התלקחות
ת.ד. 10140, מפרץ חיפה 26111 טל. 04-8469553
פקס: 04-8469320 E-mail: zilbermanj@tami-imi.co.il

To: Lilach Yakobovich - DSBG

From: Y. Zilberman - IMI

Date: 14 December 2003

Following are some of the characteristics of the products.

CONFIDENTIAL

APPENDIX VII

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH FR-1435X; DETERMINATION OF THE CONCENTRATIONS

Author

Dr. Ir. C. Brands

Study completion date

September 20, 2005

Laboratory Project Identification

NOTOX Project 419827
NOTOX Substance 146232/A

1. CONTENTS

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2. REPORT APPROVAL

PRINCIPAL SCIENTIST: Dr. Ir. C. Brands
(Analytical Chemistry)

C Brands

Date: September 20, 2005

3. INTRODUCTION

3.1. Preface

Study plan (analytical study)	Start	: 26 November 2004
	Completion	: 26 February 2005

3.2. Aim of the study

The purpose of the analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Methanol	HPLC-grade, Labscan, Dublin, Ireland
Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore Corp, Bedford, MA, USA
ISO-medium	Medium formulated using Milli-Ro water (tap water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition: $CaCl_2.2H_2O$ 293.8 mg/l; $MgSO_4.7H_2O$ 123.3 mg/l; $NaHCO_3$ 64.8 mg/l; KCl 5.8 mg/l
M2-medium	see main report

4.2. Samples

All samples not analysed on the sampling day were stored frozen. Stability of the samples under these conditions was determined during NOTOX Project 419838 "Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium". On the day of analysis, the frozen samples were defrosted at room temperature.

The samples (700 µl) were diluted in a 1:1 (v:v) ratio with methanol and thereafter ultrasonicated for 5 minutes. If necessary, the solutions were further diluted with 50/50 (v/v) methanol/ M2-medium to obtain concentrations within the calibration range.

4.3. Analysis

4.3.1. Analytical method

Quantitative analysis was based on the sum of both peaks in the HPLC chromatogram of FR-1435X (See NOTOX Project 419838: "Development and validation of an analytical method for the analysis of FR-1435X in ISO-medium").

Analytical conditions

Column	
Stationary phase	Zorbax RX-C18
Dimensions	250 x 4.6 mm; dp = 5 µm
Brand	Agilent, Palo Alto, CA, USA
Mobile phase	90/10 (v/v) methanol/Milli-Q water
Flow	1.2 ml/min
Detection	Spectrophotometric (wavelength of 225 nm)
Injection volume	100 µl

4.3.2. Calibration

On each day of analysis, calibration solutions were made up from two standard solutions of FR-1435X in methanol. The standard solutions were ultrasonicated for 15 minutes to dissolve the test substance completely and thereafter diluted with 50/50 (v/v) methanol/ISO-medium (range-finding test) or 50/50 (v/v) methanol/M2-medium (final test).

4.3.3. Injections

Calibration solutions were injected in duplicate. Procedural recovery samples and test samples were analysed by single injection.

4.3.4. Data acquisition and data processing

Data acquisition and data processing was performed using Empower software (Waters, Milford, MA, USA) version 5.00.

4.3.5. Procedural recovery samples

Standard solutions in methanol and dilutions of these solutions with methanol were used as spiking solutions. Standard solutions were ultrasonicated for 15 minutes in order to dissolve the test substance.

During the range-finding test and during the final test, blank test medium (10 ml) was spiked at concentration levels of approximately 0.025 mg/l, 0.5 mg/l and 5 mg/l [1] using spiking solutions with concentrations between 2.50 and 500 mg/l. At all concentration levels, samples were prepared and treated as described in paragraph 4.2 'Samples' (i.e. diluted in a 1:1 (v:v) ratio with methanol) and analysed. The recovery was calculated for each sample.

4.4. Interpretation

4.4.1. Calculations

All calculations were performed using non-rounded values. Rounded values are reported in the tables. Therefore, some differences might be observed when calculating the parameters as mentioned in the tables.

[1] Concentrations were not corrected for the spiking volume.

4.4.2. Formulas

General

Mean:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

where
x_i = measured value
n = number of measurements

Standard deviation

$$s_{n-1} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$$

Coefficient of variation

$$\frac{s_{n-1}}{\bar{x}} * 100\%$$

Calibration

Response: Peak area of test substance Peak 1 and Peak 2 [units]

Calibration curve: The response was correlated with the test substance concentration, using linear regression analysis (least squares method; weighting factor (1/concentration^2)).

$$R = a * C + b$$

R = response calibration solution [units]
C = concentration of test substance in calibration solution [mg/l]
a = slope [units*l/mg]
b = intercept [units]

Samples

Concentration of FR-1435X analysed in the samples:

$$C = \frac{(R - b) * d}{a} \quad [mg/l]$$

R = response sample [units]
d = dilution factor
a = slope [units*l/mg]
b = intercept [units]

Recovery of procedural recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100\ [\%]$$

Relative to nominal concentration:

$$\frac{\text{Concentration analysed}}{\text{Concentration nominal}} * 100\ [\%]$$

Relative to initial concentration:

$$\frac{\text{Concentration analysed at t = x hours}}{\text{Mean concentration analysed at t = 0 h}} * 100\ [\%]$$

5. RESULTS

5.1. Calibration curves

For each series of analysis, a calibration curve was constructed using four concentration levels. During the range-finding test one data point was excluded and during the final test two data points at the lowest concentration level were excluded. For the remaining concentration levels, two responses were used. The coefficient of correlation was in excess of 0.99 for each calibration curve.

Figure 1 shows chromatograms of a calibration solution and blank 50/50 (v/v) methanol/ M2-medium.

The difference in matrix between pre-treated samples and calibration solutions had no influence on responses and therefore on the concentrations reported.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are summarized in Table 1. Figure 2 shows chromatograms of procedural recovery samples.

Recoveries were between 70% and 110%, which indicated that analysis was performed properly.

5.2.2. Test samples

The results for the test samples are summarized in Tables 2 – 3. Figures 3 – 7 show chromatograms of blank control samples, solvent control samples, nominal 0.05 mg/l samples, nominal 5.0 mg/l samples and nominal 5.0 mg/l samples without algae.

TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration nominal [mg/l]	Concentration analysed [mg/l]	Recovery [%]	Mean recovery (Coefficient of variation) [%]
29-11-04	30-11-04 [1]	0.0250	0.0250	100	105
		0.0250	0.0275	110	
29-11-04	30-11-04 [1]	0.500	0.529	106	103
		0.500	0.500	100	
29-11-04	30-11-04 [1]	5.00	4.88	98	95
		5.00	4.59	92	(3.3)
		5.00	4.84	97	
25-02-05	25-02-05	0.0250	0.0216	87	90
		0.0250	0.0231	93	
25-02-05	25-02-05	0.499	0.508	102	103
		0.499	0.521	104	
25-02-05	25-02-05	4.99	3.52	70	85
		4.99	3.89	78	(23)
		4.99	5.37	108	

[1] Procedural recovery samples and calibration solutions were prepared and treated on 29-11-04 and stored at ambient temperature until analysis on 30-11-04, due to technical problems with the HPLC equipment.

Table 2 Concentrations of FR-1435X in test medium (range-finding test).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Nominal [mg/l]	Analysed [mg/l]	Relative to nominal [%]	Relative to initial [%]
0	22-11-04 [1]	30-11-04 [2]	0.05	0.0417	83	
			0.05	0.0369	74	
			0.5	0.288	58	
			0.5	0.286	57	
			5	2.06	41	
			5	2.37	47	
			5	2.22	44	
72	25-11-04 [1]	30-11-04 [2]	0.05	0.0234	47	59
			0.05	0.0248	50	63
			0.5	0.201	40	70
			0.5	0.198	40	69
			5	1.16	23	52
			5	1.01	20	46
			5	1.34	27	61

[1] Samples were frozen until pretreatment.

[2] Test samples were treated on 29-11-04 and stored at ambient temperature until analysis on 30-11-04, due to technical problems with the HPLC equipment.

Table 3 Concentrations of FR-1435X in test medium (final test).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Nominal [mg/l]	Analysed [mg/l]	Relative to nominal [%]	Relative to initial [%]
0	22-02-05 [1]	25-02-05	0	n.d.	n.a.	
			0	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.05	0.0414	83	
			0.05	0.0437	87	
			0.16	0.150	94	
			0.16	0.153	95	
			0.51	0.419	82	
			0.51	0.439	86	
			1.6	1.31	82	
			1.6	1.29	81	
			1.6	1.30	82	
			5.0	2.88	58	
			5.0	2.95	59	
			5.0	2.86	57	
			5.0 [3]	2.53	51	
			5.0 [3]	2.57	51	
			5.0 [3]	2.35	47	

Table 3 Concentrations of FR-1435X in test medium (final test) continued.

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Nominal [mg/l]	Analysed [mg/l]	Relative to nominal [%]	Relative to initial [%]
24	23-02-05 [1]	25-02-05	0	n.d.	n.a.	
			0	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.05	0.0139 [4]	28	33
			0.05	0.0145 [4]	29	34
			0.16	0.0199	12	13
			0.16	0.0200	12	13
			0.51	0.0569	11	13
			0.51	0.0591	12	14
			1.6	0.320	20	25
			1.6	0.285	18	22
			1.6	0.303	19	23
			5.0	0.538	11	19
			5.0	0.631	13	22
			5.0	0.644	13	22
			5.0 [3]	0.290	6	12
			5.0 [3]	0.307	6	12
			5.0 [3]	0.311	6	13
72	25-02-05	25-02-05	0	n.d.	n.a.	
			0	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0 [2]	n.d.	n.a.	
			0.05	0.0137 [4]	27	32
			0.05	0.0124 [4]	25	29
			0.16	0.0515	32	34
			0.16	0.0512	32	34
			0.51	0.171	34	40
			0.51	0.181	35	42
			1.6	0.498	31	38
			1.6	0.449	28	34
			1.6	0.530	33	41
			5.0	0.573	11	20
			5.0	0.509	10	18
			5.0	0.530	11	18
			5.0 [3]	0.708	14	29
			5.0 [3]	0.704	14	28
			5.0 [3]	0.657	13	26

[1] Samples were frozen until analysis.

[2] Solvent control.

[3] Without algae.

[4] Extrapolated value.

n.d. Not detected.

n.a. Not applicable.

FIGURES

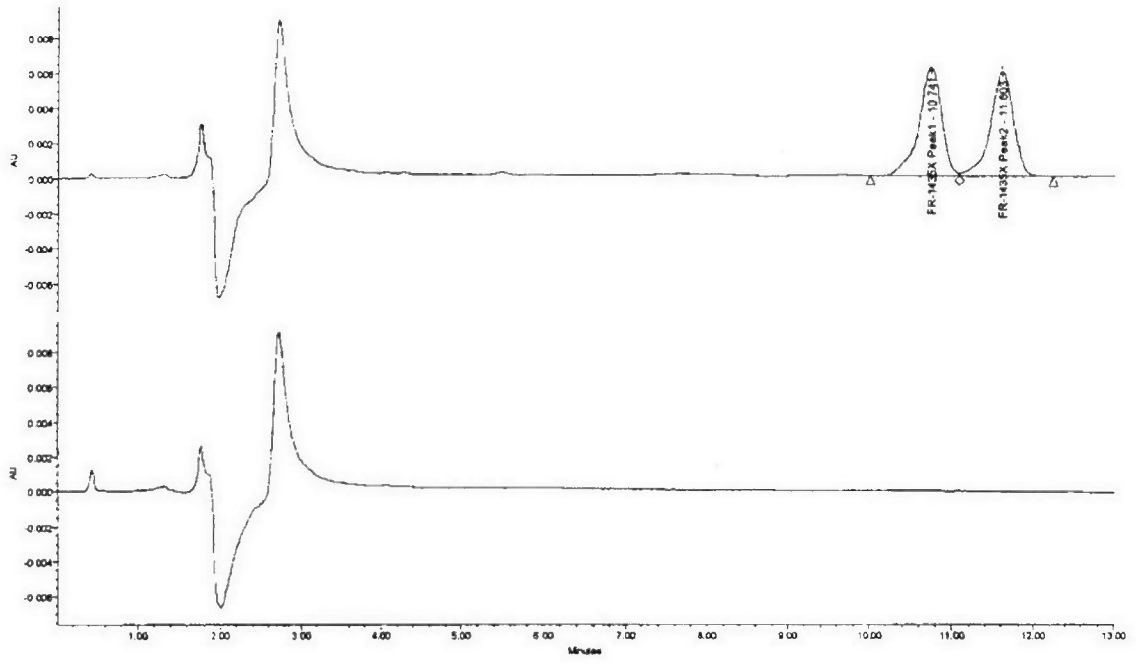


Figure 1 HPLC chromatograms of a 0.600 mg/l calibration solution (top) and blank 50/50 (v/v) methanol/M2-medium (bottom) [res.id. 2070 and 2078].

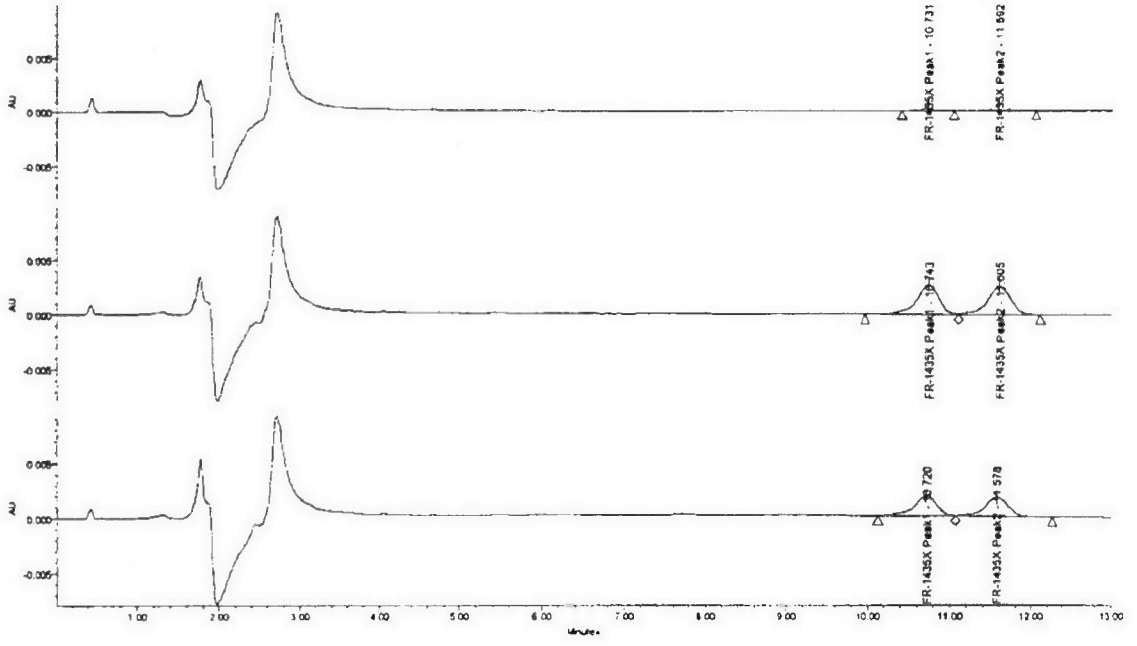


Figure 2 HPLC chromatograms of procedural recovery samples at concentrations of 0.0250 mg/l [top; res.id. 2080; dilution factor 2], 0.499 mg/l [middle; res.id. 2082; dilution factor 2] and 4.99 mg/l [bottom; res.id. 2084; dilution factor 20].

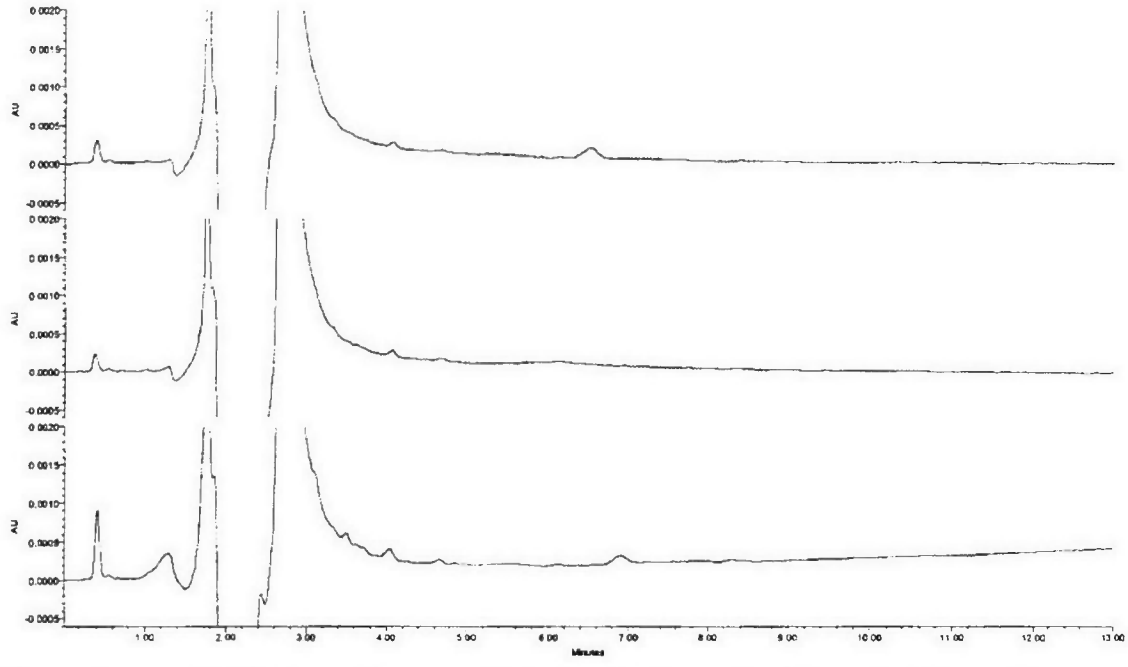


Figure 3 HPLC chromatograms of blank control samples taken at t=0 hours [top; res.id. 2116], at t=24 hours [middle; res.id. 2139] and at t=48 hours [bottom; res.id. 2089].

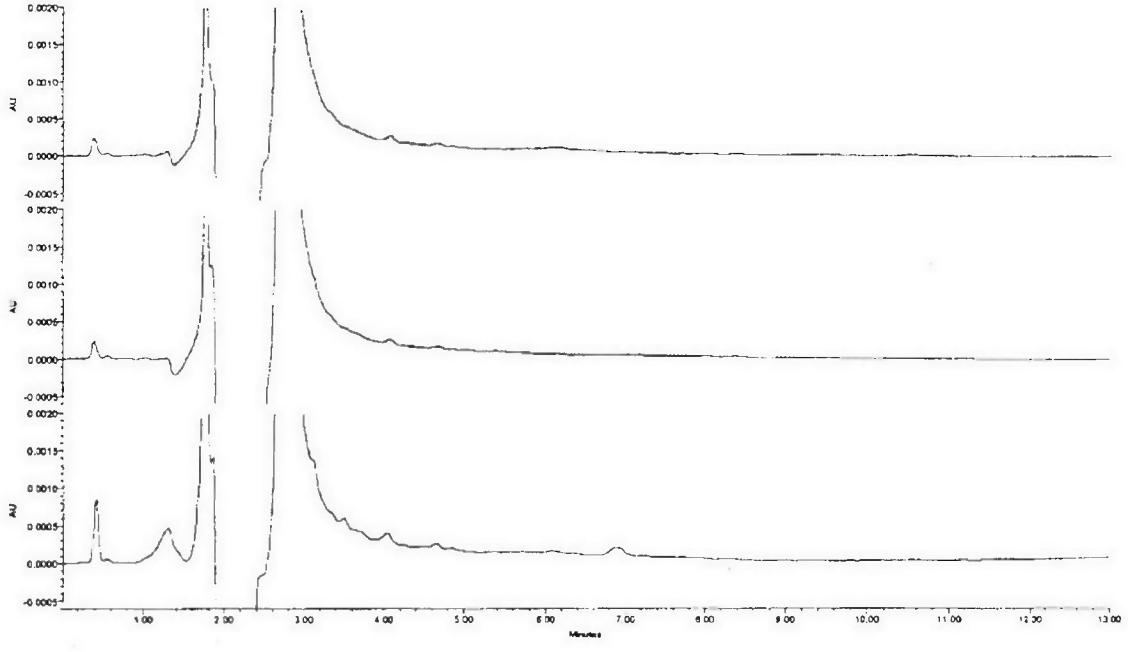


Figure 4 HPLC chromatograms of solvent control samples taken at t=0 hours [top; res.id. 2118], at t=24 hours [middle; res.id. 2141] and at t=48 hours [bottom; res.id. 2091].

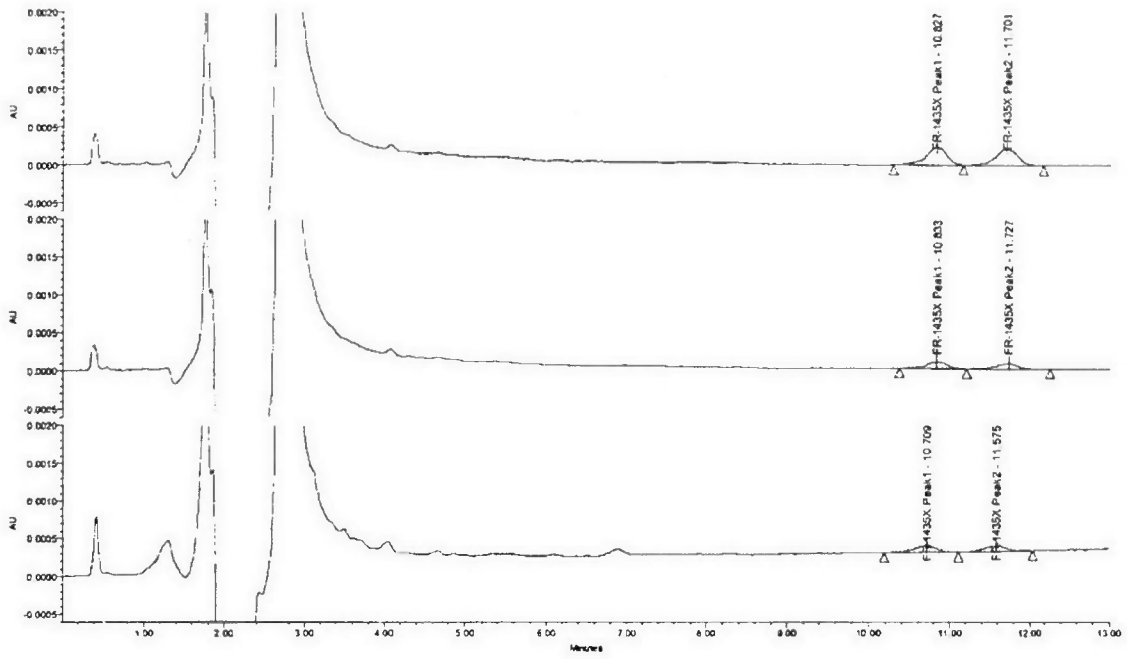


Figure 5 **HPLC chromatograms of nominal 0.05 mg/l samples taken at t=0 hours [top; res.id. 2120], at t=24 hours [middle; res.id. 2168] and at t=48 hours [bottom; res.id. 2093].**

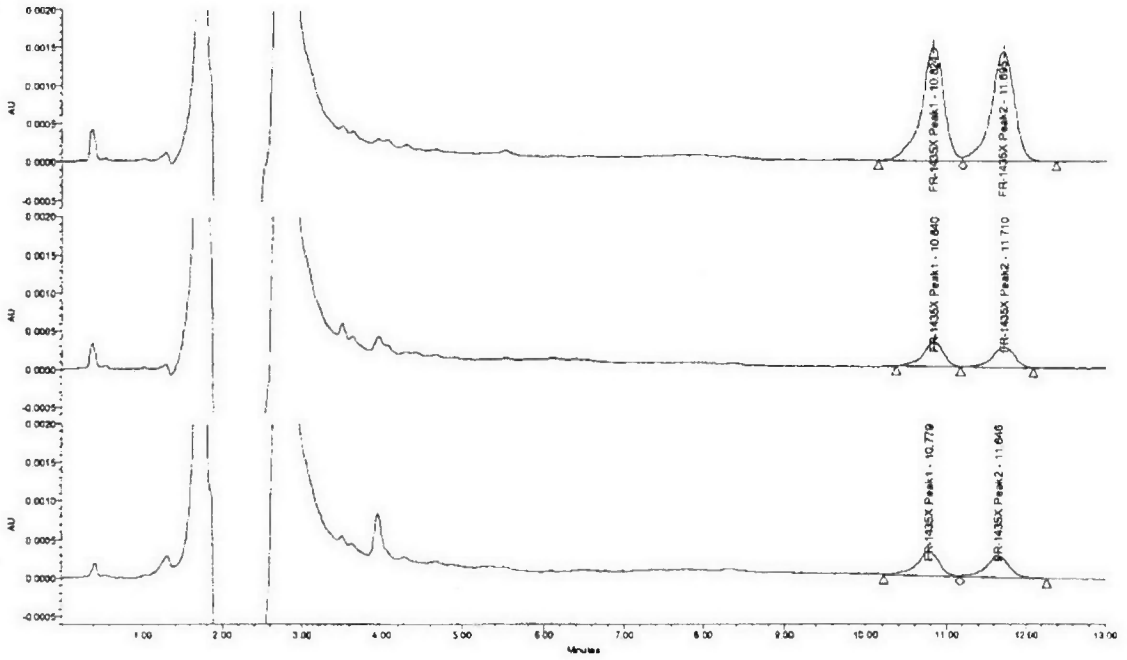


Figure 6 **HPLC chromatograms of nominal 5.0 mg/l samples taken at t=0 hours [top; res.id. 2128], at t=24 hours [middle; res.id. 2169] and at t=48 hours [bottom; res.id. 2105].**

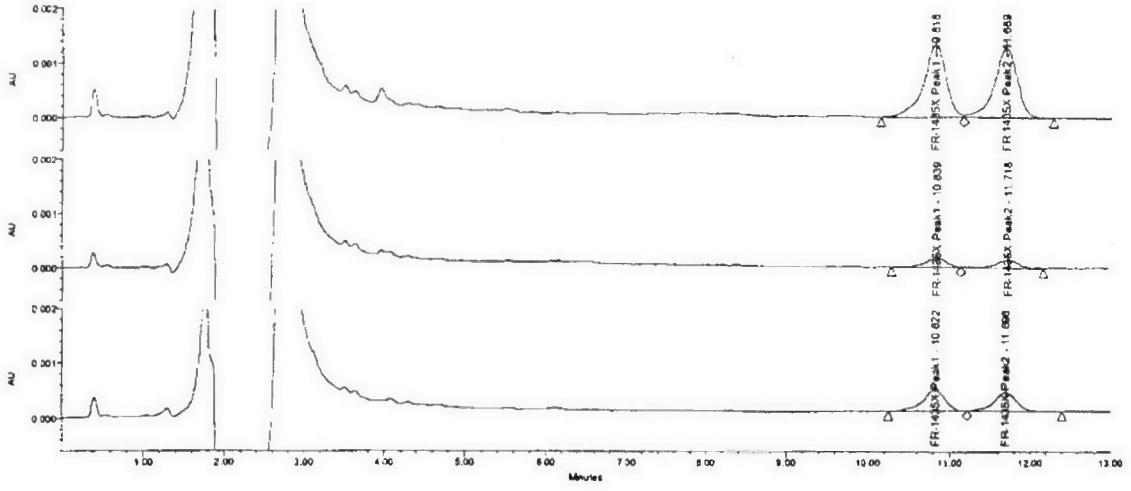


Figure 7 **HPLC chromatograms of nominal 5.0 mg/l samples without algae taken at t=0 hours [top; res.id. 2130], at t=24 hours [middle; res.id. 2153] and at t=48 hours [bottom; res.id. 2107].**

APPENDIX VIII COPY OF THE PROTOCOL + AMENDMENTS

ORIGINAL

PROTOCOL

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH FR-1435X

Author

Ir. L.M. Bouwman

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419827
NOTOX Substance 146232/A

1. CONTENTS

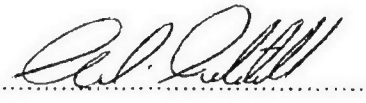
2. PROTOCOL APPROVAL

STUDY DIRECTOR: Ir. L.M. Bouwman

.....

date: 13 October 2004

HEAD OF QUALITY ASSURANCE: C.J. Mitchell B.Sc.



.....

date: 14-10-04.

SPONSOR: Dead Sea Bromine Group
Dr. S. Lifshitz

Sarit Lifshitz

.....

date: 19.10.04

3. INTRODUCTION

3.1. Preface

Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	W.J. Koolen
Principal Scientist	Dr. Ir. C. Brands
Study Plan	Start week beginning : 08 November 2004 (week 46) Completed week beginning : 13 December 2004 (week 51) Proposed Reporting date : 06 February 2005

3.2. Aim of the study

The purpose of the study is to evaluate the test substance for its ability to generate toxic effects in *Selenastrum capricornutum* during an exposure period of 72 or 96 hours and, if possible, to determine the EC_{50} for both inhibition of cell growth and reduction of growth rate for the 72-hour light period.

3.3. Guidelines

The study procedures described in this protocol are based on the ISO International Standard 8692: "Water quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*", First edition, 15 November 1989.

In addition, the procedures are designed to meet the test methods and validity criteria prescribed by the following guidelines and guidance documents:

- European Community (EC), Commission directive 2004/73/EC, Part C: Methods for the determination of ecotoxicity, EC Publication No. L152, April 2004, C-3: "Algal Inhibition Test".
- Organization for Economic Co-operation and Development (OECD), OECD guideline for Testing of Chemicals, guideline No. 201: "Algae, Growth Inhibition Test", Adopted June 7, 1984.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

3.4. Good Laboratory Practice

The study will be performed according to:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

3.5. Quality Assurance

Study and/or process inspections will be performed by the NOTOX Quality Assurance Unit to assure the GLP compliance of this study. Facility inspections are also performed at regular intervals to assure the GLP compliance of general aspects.

The protocol will be inspected to confirm that it complies with GLP regulations. The report will be inspected to confirm that the methods and results accurately and completely reflect the raw data.

3.6. Storage and retention of records and materials

Records and materials pertaining to the study, including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report, will be retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

3.7. Definitions

Cell density is the number of cells per millilitre.

Total growth or biomass is defined as the increase in total cell density over the test period. It is derived from the area under the growth curve in a linear plot. The E_BC_{50} is the concentration of test substance that results in a 50% inhibition of total cell growth relative to the control.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

If appropriate, additional definitions may be included in the report (e.g. definitions referring to poorly soluble substances).

4. MATERIALS AND METHODS

4.1. Test substance

4.1.1. Test substance

CONFIDENTIAL

Description	Amber viscous liquid (determined at NOTOX)
Batch	38278-53-4
Purity	97.2%
Test substance storage	At room temperature in the dark
Stability under storage conditions	Not indicated
Expiry date	24 September 2005 (allocated by NOTOX, 1 year after receipt of the test substance)
Density	~2000
Stability in vehicle	
Water	Not indicated
Dimethyl sulphoxide	Not indicated
Ethanol	Not indicated
Acetone	Not indicated
Safety precautions	Gloves, goggles and face mask to ensure personnel health and safety
Disposal category	IV

The sponsor is responsible for all test substance data unless determined by NOTOX.

4.1.2. Reference substance

The results of the most recent reference test with potassium dichromate (Merck, Art. 4864) will be appended to the report. This reference test will have been performed a maximum of 3 months before or after the start of this project.

4.2. Test system

Species	*Selenastrum capricornutum*
Source	In-house laboratory culture.
Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

4.3. Fresh water algae culture

Stock culture

Algae stock cultures are started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions are continuously aerated and exposed to light in a climate room at a temperature of 23 ± 2°C.

Light intensity

60 to 120 µE/m^2/s when measured in the photosynthetically effective wavelength range of 400 to 700 nm.

Stock culture medium

M1; formulated using Milli-RO water and with the following composition:

$NaNO_3$	500	mg/l
K_2HPO_4	40	mg/l
$MgSO_4.7H_2O$	76	mg/l
$Na_2CO_3.10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2.H_2O$	36	mg/l
$C_6H_5FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1.81	mg/l
$ZnCl_2$	0.11	mg/l
$CuSO_4.5H_2O$	0.08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture

3 to 4 days before the start of the test, cells from the algal stock culture are inoculated in culture medium at a cell density of 2.10^4 cells/ml. The pre-culture is maintained under the same conditions as used in the test. The cell density is measured immediately before use.

Pre-culture medium

M2; according to the ISO-Standard "Algal growth inhibition test" Nov. 1989; formulated using Milli-Q water preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2.2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	80	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l
$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2.6H_2O$	1.5	µg/l
$CuCl_2.2H_2O$	0.01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8.3 ± 0.2	

4.4. Preparation of stock and test solutions

The exact procedure for preparation of test solutions will be based on a pretest, of which the result will be kept in the raw data.

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable, and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance on the water surface or extensive precipitation, flocculation, aggregation or deposition of the undissolved fraction of the test substance. The method of preparation of the test solutions will be based on data of the test substance supplied by the sponsor and/or on the results of a preliminary test (or specific tests with the test substance performed by NOTOX when the sponsor requests these).

The method of preparation will be based on the principles laid down in the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures and referred to in the OECD Guidance Document On The Use Of The Harmonised System For The Classification Of Chemicals Which Are Hazardous For The Aquatic Environment, section 3.5: "Difficult to test substances".

The tests will be carried out without adjustment of the pH, except if pH values of the stocks are outside the range of 3-10 and are expected to induce similar extreme pH values in the test solutions.

4.5. Range-finding test

A range-finding test will be performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions will be similar to those applied in the final test with the following exceptions:
Exponentially growing algal cultures will be exposed to a range of 0.1 to 100 mg/l increasing by a factor of 10 and to a blank-control. If applicable a range will be tested up to and including the maximum solubility if this is below 100 mg/l. Three replicates are tested per concentration and three replicates in the control group. pH will at least be measured in the control and the highest test concentration.

During the exposure period the actual test concentrations should be maintained at 80% or more of the initial concentrations. In case of a 72-hour test period determination of the stability of the test substance under test conditions will be performed using samples taken at the start and at the end of the range-finding test. In case of a 96-hour study with a (suspected) photosensitive test substance determination of the stability under test conditions will be performed using samples taken at the start, after 24 hours and at the end of the range-finding test. The decision which samples will be taken for analysis is made at the start of the test in case samples need to be analysed freshly. Standard procedures assume stability of the test substance under deep freeze conditions. If stability is guaranteed, samples will be taken from all concentrations (except control(s)). The decision which samples will be analysed is then taken by the Study Director at the end of the test period based on the biological results. Optionally, sampling at the end of the test period may be limited to the concentration(s) that are chosen for analysis.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

If no toxicity is expected, based on the characteristics of the test substance or other specific information, a limit test or alternatively a limit test combined with a range-finding test will be performed.

4.5.1. Limit test

The limit test will consist of a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Test procedure and conditions will be similar to those applied in the final test except that 6 replicates will be used for both test groups. Samples for determination of actual exposure concentrations will be taken from the control and the test concentration at the start, after 24 hours and at the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium different methods of preparation may be combined in order to reach the expected water solubility.

4.5.2. Combined limit/range-finding test

In a combined limit/range-finding test, 6 replicates of exponentially growing algae will be exposed to a blank-control and a concentration of 100 mg/l or, if applicable, a saturated solution whichever is lower. Three replicates per concentration will be exposed to 0.1, 1.0 and 10 mg/l, or if applicable a range of dilutions containing 0.1, 1.0 and 10% of the saturated solution. pH will at least be measured in the control and the highest test concentration. Samples for determination of actual exposure concentrations will be taken from the control and the highest test concentration at the start, after 24 hours and at the end of the test. No further testing will be required if no effects are observed and the validity criteria are met.

Depending on the solubility of the test substance in test medium and, if appropriate the expected level of toxicity, the range of concentrations in the range-finding test may be different or include less concentrations. Alternatively, different methods of preparation may be combined in order to reach the expected water solubility (and dilutions thereof).

4.6. Final test

4.6.1. Test concentrations

Number	At least 5 concentrations in a geometric series with a factor ≤ 2.2, except when the EC_{50} is expected to be greater than the maximum concentration to be tested. In that case a limit test can be performed.
Range	Preferably, the concentration range has to cover at least one concentration causing no significant growth inhibition (preferably <10%) and a sufficient number of different concentrations causing growth inhibition >10%, with the maximum concentration inducing >50% growth inhibition to allow calculation of the EC_{50}. Maximum concentration: 100 mg/l or a saturated solution whichever is lower.
Controls	Test medium without test substance or other additives (blank) and, if relevant, a control containing test medium without test substance but treated in the same way as the test substance solutions.
Replicates	3 replicates of each test concentration or 6 replicates in case of a limit test, 6 replicates of the controls,

	1 replicate without algae at the highest test concentration, and, if relevant, 1 replicate of each test concentration without algae (turbidity control). If relevant, one or more extra replicates for sampling purposes.

4.6.2. Test procedure and conditions

Test duration	At least 72 hours
Test type	Static
Test vessels	100 ml, all-glass
Medium	M2
Cell concentration	An initial cell density of 1 x 10^4 cells/ml using an exponentially growing preculture.
Illumination	Continuously using TLD-lamps of the type 'Cool White' of 30 Watt with a light intensity within the range of 60 to 120 $\mu E.m^{-2}.s^{-1}$, not varying by more than 20%.
Temperature of medium	21-25 °C, constant within 2°C.
pH	Between 6.0 and 9.0 Should not vary by more than 1,5 unit at the end of the test in any test solution. However, if the pH at the end of the test period had increased above 9.0 and/or varies by more than 1.5 unit and this increase is solely related to a relatively high rate of algal growth, this will be accepted.
Incubation	Vessels are distributed at random in the incubator. During incubation the algal cells are kept in suspension by continuous shaking, thereby improving gas exchange and reducing pH variation in the test solutions.

4.6.3. Sampling for analysis of test concentrations

Frequency	At the start and the end of the test. If the test concentrations are expected to be unstable, extra samples will be taken after 24 hours following exposure.
Concentrations (standard[1])	Samples will be taken from at least three concentrations, i.e. the lowest, a middle and the highest, and the control(s) for analysis. At the start of the test care will be taken not to include any floating layer, test substance film or undissolved material in separate vessels. At the end of the test samples will

[1] The standard frequency of sampling is only applicable provided that test solutions are diluted using one stock and test concentrations should be above 1 mg/l. Sampling will include all test solutions if the previous mentioned conditions are not met or if the Study Director decides this is essential for other reasons. Alternatively, sampling may be limited to those test solutions that are biologically relevant.

	be taken from the approximate centre of the pooled solutions of the vessels containing the algal suspensions at each concentration.
Number of samples	Sampling will consist of singular samples per treatment. Should the analytical validation require duplicate or multiple samples per treatment, this will be followed without prior notification.
Volume	Standardly, volumes of 700 µl will be taken, but depending on the limit of detection of the analytical method used in relation to the test concentrations the volume may differ.
Storage	If stability of test concentrations under deep-freeze conditions is ensured, the samples will be stored in a deep-freezer until analysis. Optionally, samples can be stored under different conditions (e.g. room temp. or in refrigerator) if stability under these conditions is ensured.
Extra samples	In case singular samples are taken, which are known to be stable under the storage conditions, extra samples will be taken and stored for possible analysis until delivery of the final report with a maximum of three months.
Quality control	Compliance with the quality criteria regarding maintenance of actual concentrations will be demonstrated by running a test vessel at the highest substance concentration but without algae and samples for analysis will be taken at the start and the end of the test period.
Analyses	When used for analysis the entire volume of each sample will be taken for further dilution or pre-treatment. The analytical method used will be based on the results of a separate project for the development and validation of the analytical method. If study specific adjustments of the analytical method or sample pre-treatment procedures are necessary, these will be developed and tested before the performance of the final test. Detailed specification of these additional analytical procedures will be put down in a protocol amendment (see also 'Additional procedures').

4.6.4. Measurements and recording

pH	At the beginning and at the end of the test in at least one vessel per concentration. The pH of the solution should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel.
Cell densities:	
Clear solutions	At the beginning of the test, cell density is based on counting by microscope using a counting chamber. Thereafter cell densities are determined daily by spectrophotometric measurement of samples at 720 nm using a Varian Cary 50 single beam spectrophotometer with immersion probe (path length =20 mm). Varian Nederland BV., Houten, The Netherlands. Algal medium will be used as a blank.
Turbid solutions	If the test solutions are so turbid that they disturb the spectrophotometric measurements substantially, the algal densities will be recorded by direct counting using a microscope.
Coloured solutions	If the test solutions are increasingly coloured to an extent that significant absorption of wave lengths necessary for normal algal growth may be expected, this will be examined in a separate range-finding test. In this test algal suspensions will be exposed to light filtered through test substance solutions at relevant concentrations.

4.7. Interpretation

4.7.1. Acceptability of the test

1. The cell density in the control cultures should have increased by a factor of at least 16 within three days.
2. Test conditions (temperature, pH) will be maintained within the limits prescribed by this protocol.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

4.7.2. Additional procedures

Additional or alternative procedures will be required for the testing of:

1. Volatile substances;
2. Very toxic or low soluble substances (test concentrations < 1 mg/l);
3. Hydrolytically unstable or photosensitive substances;
4. Dye stuffs;
5. pH affecting substances;
6. Substances that are not stable under deep-freeze conditions.

These additional procedures may require the amending of:

1. Preparation of test solutions;
2. The frequency of sampling and analysis for the determination of actual test concentrations;
3. Extension of the analytical program with respect to sample treatment and the sensitivity of the analytical method;
4. In case of a dye stuff: additional testing to compare the effect on algal growth induced by the color of the test solutions with the results of the toxicity test.
5. If applicable, additional testing with pH adjustment.

The additional procedures are no part of the standard test procedures and will be applied only after emission of an authorised protocol amendment. In such a case the amended procedures will be effective only after NOTOX has received any kind of authorisation from the study monitor.

4.7.3. Data handling

Defining exposure concentration

1. The results will be based on the nominal or initial (if not in agreement with nominal) test substance concentrations if the analytical program has confirmed that the measured test substance concentrations remained within 20% of the nominal or initial concentrations.
2. If the deviation of the exposure concentrations of the test substance is greater than ± 20% of the nominal or initial concentrations, the results will be expressed in terms of average exposure concentrations. Where measured data are available for the start and end of the test, these concentrations are geometric means calculated from the concentrations measured at the start and end of the test. In case that additional analysis is performed after 24 hours, a time weighed average concentration is calculated. Where at the end of the test measured concentrations are below the analytical detection limit, such concentrations shall be considered to be half that detection limit.

Calibration curve

At the start of the algal test, a calibration curve will be made using a minimum of six dilutions of one or two of the pre-cultures. The software of the Cary-50 Spectrophotometer will be used to plot cell density against extinction. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of areas under the growth curves

The area below the growth curve is calculated using the following formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2 \times N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2 \times N_0}{2} \times (t_n - t_{n-1})$$

Where: A = area
N_0= nominal number of cells/ml at the start of the test
N_1= measured number of cells/ml at t_1
N_n= measured number of cells/ml at t_n
t_1= time of first measurements after beginning of the test
t_n= time of n^{th} measurement after beginning of the test

The percentage inhibition of the cell growth at each test concentration (I_T) is calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} \times 100$$

Where: A_C = area below the growth curve obtained in the control
A_T = area below the growth curve at each test substance concentration

Growth inhibition will be calculated for the total period of exposure.

Comparison of growth rates
The average specific growth rate (μ) for exponentially growing cultures is calculated as:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n}$$

The average growth rate at each test substance concentration is then compared with the control value and the percentage reduction in growth rate is calculated.

Determination of the effect parameters (NOEC, EC_{50}, EC_{10})
The percentages of growth inhibition and/or the percentages of growth rate reduction will be set out against the logarithms of the corresponding concentrations of the test substance. If a test concentration related effect is present, calculation of the EC-values will be based on the respective curves corresponding to the various observation times using regression analysis. If possible, also the EC_{10} will be determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993. Optional: data will be analysed using the DEBTOX-model for generation of NEC and EC_x-values as described in 'The Analysis of Aquatic Toxicity Data' by S.A.L.M. Kooijman and J.J.M. Bedaux, 1996.

The NOEC will be based on statistical analysis of the data. Data obtained for the test concentrations will be compared with those obtained in the solvent or negative control using TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman.

Optionally, other statistical analyses may be performed if appropriate.

5. DISTRIBUTION

Original: Study Director
1 Copy: Technical Coordinator
1 Copy: Analytical Chemistry
1 Copy: QAU/Management
1 Copy: Sponsor

ORIGINAL

PROTOCOL AMENDMENT NO : 1

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Dead Sea Bromine Group Purchasing Division of DSBG P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

1. Page 6, paragraph 4.1.1. Test substance information:

Description	Brown liquid

REASONS FOR AMENDMENT

1-2 The data has been updated according to information of the sponsor, dated January 06, 2005.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

12 January 2005
.....
date:

PROTOCOL AMENDMENT NO : 2

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

1. The final test will be performed with the following test conditions:
   - Test concentrations in silanised glassware: 0.05, 0.16, 0.51, 1.6 and 5.0 mg/l.
   - Samples for analysis will be taken at the start, after 24 hours of exposure and at the end of the test.
2. The final test will be performed in week 8. The proposed reporting date will be 10 April 2005.

REASONS FOR AMENDMENT

1 and 2: Analytical results obtained during the range-finding test with algae showed that measured concentrations could not be maintained stable during the test period.

APPROVAL
Study director

Ir. L.M. Bouwman — date: 01 February 2005

Sponsor

Sarit Lifshitz

Dr. S. Lifshitz — date: 6/2/05

PROTOCOL AMENDMENT NO : 3

ORIGINAL

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

During the final flow-through test duplicate or triplicate samples will be taken from the control and all test concentrations.

REASONS FOR AMENDMENT

Duplicate or triplicate samples are required for the analytical validation.

APPROVAL

Study director

Ir. L.M. Bouwman — date: 03 February 2005

Sponsor

Sarit Lifshitz

Dr. S. Lifshitz — date: 6/2/05

ORIGINAL

PROTOCOL AMENDMENT NO : 4

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compound Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

1. Page 4, paragraph 3.1. Preface:

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel

REASONS FOR AMENDMENT

1. The data has been updated according to information of the sponsor, dated January 31, 2005

APPROVAL
Study director

.....
Ir. L.M. Bouwman

10 February 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 5

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

The final test is a static test.

REASONS FOR AMENDMENT

Protocol amendment no 3 inadvertently states a flow-through test.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

23 February 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 6

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

1. The EEC guideline should read:
   - European Economic Community (EEC), EEC Directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, C-3: "Algal Inhibition Test", Adopted December 1992.

REASONS FOR AMENDMENT

1. Correction of the text in the protocol.

APPROVAL
Study director

.....
Ir. L.M. Bouwman

07 April 2005
.....
date:

ORIGINAL

PROTOCOL AMENDMENT NO : 7

Study Title	Fresh water algal growth inhibition test with FR-1435X
Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. S. Lifshitz
NOTOX Substance	146232/A
NOTOX Project	419827

AMENDMENT DESCRIPTION

1. Page 6, paragraph 4.1.1. Test substance information:

Density	~2000 kg/m^3

REASONS FOR AMENDMENT

1. The data has been added according to information of the sponsor, dated 15 August 2005 This has no effect on other data.

APPROVAL

Study director

.....	26 August 2005 .....
Ir. L.M. Bouwman	date:

- Attachment 18 -

REPORT

Study Title

BIOCONCENTRATION TEST IN RAINBOW TROUT WITH [U-PHENYL-^{14}C] FR-1435X (FLOW-THROUGH)

Author

Ir. L.M. Bouwman

Study completion date

14 December 2005

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419849
NOTOX Substance 148797/A

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

Tap water and Nutra analyses were not performed under GLP and therefore excluded from this GLP statement.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: .....11. December 2005.....

Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology

Date: .....15/12/2005.....

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	15-Mar-05	15-Mar-05	15-Mar-05
	Amendment 1 of protocol	29-Mar-05	29-Mar-05	29-Mar-05
	Amendment 2 of protocol	30-Mar-05	30-Mar-05	30-Mar-05
	Exposure	01-Apr-05	01-Apr-05	01-Apr-05
	Analysis	01-Apr-05	01-Apr-05	01-Apr-05
	Amendment 3 of protocol	05-Apr-05	05-Apr-05	05-Apr-05
	Report	03-Aug-05	03-Aug-05	03-Aug-05
	Amendment 4 of protocol	09-Aug-05	09-Aug-05	09-Aug-05
Process	**Environmental toxicology**	18-Apr-05	22-Apr-05	22-Apr-05
	Test substance handling			
	Exposure			
	Observations/Measurements			

Head of Quality Assurance
C.J.Mitchell B.Sc.

Date: ...15-dec-05....

4. SUMMARY

Fish bioconcentration test in rainbow trout with [U-phenyl-^{14}C] FR-1435X.

The study procedure described in this report was based on the OECD guidelines for Testing of Chemicals, guideline No. 305, 1996 and EC directive Annex IIID, part C.13, 1998 (replicate of OECD no. 305).

The specific activity of the batch of [U-phenyl-^{14}C] FR-1435X used was 29.12 mCi/mmol, the radiochemical purity was 99.2% (TLC) and the chemical purity was ≥ 98%. Solubility in water is 0.47 mg/l with an octanol-water partition coefficient (Log P_{ow}) of 5.6. The estimated uptake phase for [U-phenyl-^{14}C] FR-1435X until the steady state in fish (90%) has been reached was estimated to be 16 days (SSt_{90}).

Owing to the low solubility of [U-phenyl-^{14}C] FR-1435X in water two stock solutions were prepared in acetone. The stock solutions were dosed via a computer-controlled system consisting of micro-dispensers into a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter at a flow rate of 20 l/h. In the mixing flask the dosed stock volume and the dilution water were mixed under continuous stirring at a ratio of 1 : 100,000. The final target concentrations were 0.50 and 5.0 µg/l.

At the start of the Bioconcentration study 44 fish per concentration were exposed to 0.50 and 5.0 µg [U-phenyl-^{14}C] FR-1435X per litre and 22 fish were exposed to the control. The uptake phase lasted for 32 days, during which samples were taken from the test medium (for quantitative and qualitative analysis) at six time points and from the fish at five time points. During the subsequent depuration phase, which lasted for 14 days fish were sampled at days 32 (same sample as last day uptake phase), 34 and 41.

The mean Time Weighed Average (TWA) concentrations of [U-phenyl-^{14}C] FR-1435X in test medium at nominal 0.50 and 5.0 µg/l were 0.52 ± 0.023 µg/l and 4.9 ± 0.16 µg/l, respectively. The measured concentrations varied within the ± 20% window of the mean TWA concentration at both 0.50 and 5.0 µg/l.

The purity of [U-phenyl-^{14}C] FR-1435X in the stock solutions was > 97% during the uptake phase. The purity of [U-phenyl-^{14}C] FR-1435X in the nominal test solutions of 0.50 and 5.0 µg/l were 77 ± 1.4% and 83 ± 7.4% during the main part of the test, respectively. The percentage of [U-phenyl-^{14}C] FR-1435X in residual water of the 0.50 and 5.0 µg/l concentrations after extraction with DCM were 20 ± 2.4% and 18 ± 2.1%, respectively. This indicated that one or more metabolites were formed during the test.

The steady state concentration in fish was reached after 6 days of exposure. The mean concentrations of [U-phenyl-^{14}C] FR-1435X in fish at nominal 0.50 and 5.0 µg/l from day 6 until day 32 were 0.40 ± 0.032 µg/g and 4.2 ± 0.43 µg/g, respectively. The measured concentrations varied within the ± 20% window of the mean concentration in fish at both 0.50 and 5.0 µg/l.

The curves for depuration showed an exponential decrease of [U-phenyl-^{14}C] FR-1435X in the fish at both levels of exposure. After 9 days of depuration the concentrations in the fish were less than 3% of the concentrations at the start of the depuration phase (2-3%).

Combining the results at the two concentrations tested the BCF_{ss} was 803 ± 75 and the BCF_k was 852 ± 114. The DT_{50} value for depuration of [U-phenyl-^{14}C] FR-1435X was 1.8 days.

Based on these results [U-phenyl-^{14}C] FR-1435X appears to be only slightly accumulating in fish combined with a highly efficient excretion by the fish. Hence there is no substantial risk for bioconcentration of FR-1435X in fish.

5. INTRODUCTION

5.1. Preface

Sponsor	Bromine Compounds Ltd. P.O. Box 180 BEER-SHEVA 84101 Israel
Study Monitor	Dr. O. Manor
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Principal scientist	Dr. Ir. H. Willems
Technical Coordinators	Ing. S. Buitenweg Ing. W. Thijssen
Study Plan	Start : 01 April 2005 Completion : 17 May 2005

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to accumulate in tissues of Rainbow trout (*Oncorhynchus mykiss*) during an initial uptake period of 30 days and a depuration period of maximum 60 days.

5.3. Guidelines

The study procedure described in this report was based on the OECD guidelines for Testing of Chemicals, guideline No. 305,: "Bioconcentration: Flow-through Fish Test", June 1996 and EC directive Annex IIID, part C.13:."Bioconcentration: flow-through fish test", 1998 (replicate of OECD no. 305).

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

5.5. Definitions

Uptake phase is the period during which fish are exposed to at least two different sublethal concentrations of the test substance and samples are taken to examine uptake of test substance.

Depuration phase is the period following the uptake phase, when fish have been transferred to clean, untreated water. Samples are taken to examine the decrease of test substance concentrations in the fish. A depuration phase is not needed if uptake has been insignificant (e.g. the BCF is less than 10).

Flow-through system is a system where test substance is dosed every 2 or 10 minutes and mixed with a continuous flow of dilution water to provide continuous renewal of the test solutions.

A steady-state is defined as the moment when the curve in the plot of test substance in fish (C_f) against time becomes parallel to the time axis and three successive analysis of C_f made in samples taken at intervals of at least two days are within ± 20% of each other.

The 'steady-state' bioconcentration factor is calculated both as the ratio (BCF_{ss}) of concentration in the fish (C_f) and in the water (C_w) at apparent steady-state and as a kinetic bioconcentration factor (BCF_k) defined as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first-order kinetics. The BCF is expressed as a function of the total weight of the fish.

LSC stands for liquid scintillation counting and is a measure for the radioactivity in samples based on the activation of fotons in a scintillation liquid.

6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Radio labeled test substance

[ ]

CONFIDENTIAL

[ ]

Description	Colourless oil
Batch	SEL/1489A
Radiochemical purity	97.2 %(HPLC) 99.2 %(TLC)

Chemical purity	~99% (NMR), 98.0% (HPLC)
Test substance storage	In the freezer in the dark
Stability under storage conditions	Not indicated
Expiry date	26 January 2006 (allocated by NOTOX, 1 year after receipt of the test substance)
Specific activity	29.12 mCi/mmol (1.70 MBq/mg)
Total activity	185.6 MBq + 196.7 MBq
Supplier	Scynexis Ltd, UK
Date received	January 26, 2005

The radiochemical purity was determined by the supplier and was also checked at NOTOX using Thin Layer Chromatography (TLC) before the start of the experiment.

6.1.2. Additional information on test substance

a. Solubility in water is 0.47 mg/l.
b. Octanol-water partition coefficient, Log P_{ow} is 5.6.
c. Stability in water: No information available
d. Test substance is not readily biodegradable.
e. Volatility: Not applicable under standard conditions
f. LC_{50} (96h) for carp: > water solubility

6.2. Test system

Species	Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1988)
Source	Trout Hatchery Blitterswijk, The Netherlands
Initial fish weight	3.71 ± 0.67 g
Initial fish length	6.2 ± 0.37 cm
Characteristics	Healthy fish supplied with a health certificate.
Reason for selection	The system has been selected as an internationally accepted species.
Total fish used	110

6.3. Holding

Quarantine/Acclimatisation	At least 12 days after delivery.
Medium	Tap-water (Hardness 180-200 mg $CaCO_3$/l; see also Appendix IV).
Measurements	pH, nitrate and nitrite concentration and ammonia concentration: once a week. Temperature: every day.
Feeding	Daily with pelleted fish food.
Validity of batch	In the batch of fish used for the test, mortality during the seven days prior to the start of the test was less than 5%.

6.4. Prediction of the duration of the test

Based on the octanol-water partition coefficient (Log P_{ow}) of [U-phenyl-^{14}C] FR-1435X, the uptake phase until the steady state has been reached was estimated by the empirical relationship of the depuration rate constant (k_2) and the Log P_{ow}: $Logk_2 = -0.414 \log(P_{ow}) + 1.47$ (according to the OECD Guideline). The uptake phase for [U-phenyl-^{14}C] FR-1435X until the steady state (90%) has been reached was estimated to be 16 days.

6.5. Test concentrations

[U-phenyl-^{14}C] FR-1435X	0.50 and 5.0 µg/l
Control	Test medium without test substance but including the additive acetone used as carrier solvent.

6.6. Preparation of stock solutions

The standard test procedures require generation of test solutions, which contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system will be prevented or avoided, e.g. film of the test substance on the water surface or extensive precipitation, flocculation or deposition of the undissolved fraction of the test substance.

Owing to the low solubility of [U-phenyl-^{14}C] FR-1435X in water stock solutions were prepared in acetone (HPLC grade, Labscan Ltd, Dublin, Ireland). The concentrations in these stocks were a factor 100,000 higher than the final concentration in water, i.e. 50 and 500 mg/l, respectively in the stock solutions.

6.7. Dosing system

The stock solutions were dosed via a computer-controlled system consisting of micro-dispensers (Gilson). Via this system the dosed volume from the stock entered a mixing flask separately from the tap water supply. The tap water was supplied via a flow meter and the flow rate was 20 l/h. In the mixing flask the dosed stock volume and the dilution water were mixed under continuous stirring at a ratio of 1 : 100,000. The whole system was checked daily.

6.8. Bioconcentration test

6.8.1. Specific conditions

Test duration	46 days, i.e. an uptake phase of 32 days and a depuration phase of 14 days
[U-phenyl-^{14}C] FR-1435X	Both test concentrations consisted of [U-phenyl-^{14}C] FR-1435X with expected activities of 51 dpm/ml (0.50 µg/l) and 510 dpm/ml (5.0 µg/l).
Number of fish	44 fish per test group and 22 in the control.

6.8.2. Sampling for analysis of test substance concentrations in fish and water

Table 1 shows the schedule for the water and fish sampling during the bioconcentration test.

Table 1 **Sampling schedule for the bioconcentration test with [U-phenyl-14C] FR-1435X**

Uptake phase	Frequency (days)	No. of water samples			No. of fish per sample		
		Control	0.50 µg/l	5.0 µg/l	Control	0.50 µg/l	5.0 µg/l
Check	-1	3	3	3			
Start	0	3	3	3			
1st	3	3	3	3	2	4	4
2nd	6	3	3	3	2	4	4
3rd	10	3	3	3	2	4	4
4th	20	3	3	3	2	4	4
5th	32	3	3	3	2	4	4
Depuration phase							
6th	34		-		2	4	4
7th	41		-		2	4	4

6.9. General test procedure and conditions

Test design	Flow-through.
Introduction of fish	After test concentrations were allowed to stabilise for at least 12 hours following the start of the dosing.
Test vessels	Uptake phase: ca. 100 litre, stainless steel Depuration phase: ca. 30 litre, glass
Maximum loading	0.34 g fish/litre/day
Illumination	16 hours photoperiod daily
Aeration	Aeration was introduced after 1 day of exposure and was maintained until the end of the test period.
Feeding	Nutra (see Appendix V for composition). Ration was 1-2% of body weight per day. Recalculation of the feeding rate was performed after each sampling point (fish samples).
Cleaning	Uneaten food and fecal debris were siphoned daily from the test chambers about an hour after feeding.
Euthanasia	The fish sampled were instantly killed by a blow on the head followed by cervical incision. At the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.

6.10. Treatment of water samples for quantitative analysis

Method of sampling	Test solutions were sampled from a central point in the test chamber using mechanical pipettes with disposable tips.

First water samples	Taken from the control and both vessels containing the test concentration and analyzed before introduction of the fish to check if the actual test concentrations were in agreement with the expected concentrations.
Volume	10 ml
Storage	Samples were measured on the day of sampling
Quantitative measurement	Counting on a Liquid Scintillation Counter (TR-1900, Packard, Meriden, CT, USA) after addition of an appropriate amount of Ultima XR cocktail (Packard Bioscience B.V., Groningen, The Netherlands) to the water samples.

6.11. Treatment of water samples for qualitative analysis

Sampling frequency	At the start (day 0) and weekly thereafter, samples of 300 ml were taken from both test concentrations to perform qualitative analysis of [U-phenyl-^{14}C] FR-1435X in the test solutions.
Handling	The water samples were extracted with dichloro-methane (DCM; HPLC grade, Labscan) and the extract was analyzed with Thin Layer Chromatography (TLC).
TLC conditions	Si-60 TLC plate; mobile phase: toluene/tert-buthylmethylether 40:1 (p.a, Merck/Uvasol, Merck).

6.12. Treatment of fish samples

Handling	The fish sampled were rinsed quickly with untreated water, blotted dry, instantly killed by a blow on the head followed by cervical incision and then weighed.
Homogenisation in Soluene	Per sampling point fish were pooled in two samples of two fish each, except for the control for which there was one sample containing two fish. Fish tissues were dissolved in Soluene 350 overnight (10 ml per gram tissue) at 60°C. Thereafter samples corresponding with ca. 200 mg fish were transferred to LSC-vials and bleached with 30% H_2O_2 (200 µl per vial). The vials were incubated for an additional period of at least 2 hours at 60°C. Subsequently, the scintillation liquid was added to each vial and samples were then left to stabilize for 5-23 hours before measurement.
Quantitative measurement	Determination of the concentrations of ^{14}C-labeled substance in fish homogenates was performed by LSC. Radioactivity in the samples was determined by counting an aliquot in an appropriate amount of Hionic Fluor cocktail (Packard Bioscience B.V., Groningen, The Netherlands) on a Liquid Scintillation Counter.

6.13. Further measurements and recording

Fish appearance and behavior	Although the test concentration was not expected to induce toxic effects, fish were observed daily for any adverse effects.
Dissolved oxygen content and pH	Prior to the introduction of the fish and once a week during the uptake and depuration periods.
Temperature of medium	Continuously in each vessel starting from one day before the introduction of the fish.
Total hardness and conductivity	At the start and the end of the uptake period and at the end of the depuration period.

6.14. T(D)OC analyses

Frequency of sampling	Samples for analysis of Total (Dissolved) Organic Carbon were taken from the control once a week starting one day before introduction of the fish.
Sample volume	40 ml
Extra samples	Extra samples of 40 ml were taken and stored for possible analysis until delivery of the final report with a maximum of three months.
Apparatus	Duplicate samples were injected into a Shimadzu TOC-V_{CPH} total organic carbon analyzer combined with a Shimadzu ASI-V auto sampler (Shimadzu Benelux, The Netherlands). Each sample was analysed in triplicate. DOC values, determined as the Total Organic Carbon (TOC) values in the pre-treated solutions, were measured as the difference between the TC and IC analysis values.
Storage of samples	All samples were stored in a freezer until analysis.
Calibration solution(s)	TC standard: Potassium hydrogen phtalate solution ($C_8H_5KO_4$, Nacalai tesque Inc., Kyoto, Japan). IC standard: Sodium hydrogen carbonate ($NaHCO_3$, Nacalai tesque Inc., Kyoto, Japan) / sodium carbonate solution (Na_2CO_3, Nacalai tesque Inc., Kyoto, Japan).

6.15. Interpretation

6.15.1. Data handling

The Time Weighed Average (TWA) concentration in water was calculated by:

$$TWA = \sum_{n=1}^{m} \frac{t_{n+1} - t_n}{t_{n+1}} \sqrt{C_n \times C_{n+1}}$$

with C_n and C_{n+1} being the concentrations measured on the successive days of sampling t_n and t_{n+1} with n being the number of samples starting at 1 up to *m*, the last taken sample taken during the uptake phase.

Evaluation of the results started with obtaining the uptake curve by plotting the measured concentrations in/on fish and/or lipid fraction against time on arithmetic scales. If the curve has reached a 'plateau' the BCF_{SS} was calculated from:

$$BCF_{ss} = \frac{\overline{C}_{f_{ss}}}{\overline{C}_{w_{ss}}} \left(\frac{= Mean\ concentration\ of\ test\ subst.\ in\ fish\ at\ steady\ state}{= Mean\ concentration\ of\ test\ subst.\ in\ water\ at\ steady\ state} \right)$$

The kinetic BCF (BCF_k) was calculated as the ratio k_u/k_d, the two first-order kinetic constants. The k_u is defined as the uptake rate constant, while the k_d is determined from the depuration curve. Data will be interpretable where variation in uptake/depuration constants between the two test concentrations is less than 20%. The K_d is defined as the slope of the depuration curve and the estimated uptake rate was calculated from:

$$\frac{\Delta C_f/\Delta t + (k_d * C_f)}{C_w}$$

Where: C_f = concentration in fish at a certain time point
C_w = TWA concentration in water at a certain time point
ΔC_f = concentration increase over a certain time period
Δt = time period

6.15.2. Acceptability of the test

1. Temperature variation was less than ± 2°C during the major part of the test.
2. The oxygen concentration was maintained at at least 60% of the saturation value throughout the test (> 6 mg/l at 15 °C).
3. The analytical results showed that the variation of the actual test substance concentrations was maintained within ± 20% of the mean of the measured values during the uptake phase.
4. The TOC present in the water during the uptake phase did not exceed 10 mg/l.

6.16. List of protocol deviations

1. The initial fish length was 6.2 ± 0.37 cm and therefore slightly exceeding 6 cm given in the protocol but still below the maximum limit of 12 cm according to the guideline.
2. The maximum fish loading was 0.34 g/l/day (44 fish x 3.71 g / (20 l/h x 24 h)), and therefore exceeding 0.1 g/l/day given in the protocol but still below the maximum limit of 1 g/l/day according to the guideline.
3. Recalculation of the feeding rate was performed after each sampling point (fish samples) instead of every week since fish were weight at each sampling point.
4. Fish samples were bleached with 30% H_2O_2 instead of 35% H_2O_2. The percentage mentioned in the protocol was not correct.
5. TOC samples were frozen until analysis. The TOC concentration of test water is not considered to change by this procedure.
6. During the depuration phase TOC was > 10 mg/l at two out of three sampling points.
7. At several time points during the test temperature was not measured due to the fact that the temperature sensors were removed when the vessels were cleaned and because of short-time disfunction of the sensors. There were no reasons to assume temperature deviated significantly from the range measured during the main part of the study.

8. At day 31 (uptake phase) temperature was 11.1°C at the 0.50 µg/l concentration, which is below the optimum temperature for Rainbow trout (12-17°C, constant within 1°C). This was only for a relatively short period and there were no effects observed on the fish.
9. Extra oxygen measurements were performed at day 0 and day 1 to check if oxygen levels remained above 60% of saturation. This resulted in extra information and due to the observed drop in oxygen concentration (from 10.3-10.5 to 7.5-8.8 within 1 day), it was decided to use continuous aeration in order to prevent oxygen levels from dropping below the optimum range.
10. Fish sampled during the test were instantly killed by a strike to the head followed by cervical incision. In addition, at the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water. This standard procedure was not mentioned in the protocol.
11. After addition of Hionic-Fluor to the fish samples taken on days 3, 6 and 10 samples were left incubating for a period < 16 hours. Since the Hionic-Fluor control was between 15-40 cpm, measurements were acceptable.
12. At day 3 fish samples taken from the control vessel showed a relatively high radioactivity. Re-analyses showed the same results. Since, the results of the Hionic-Fluor control was within the range of 15-40 cpm the results of the fish samples were accepted.
13. Inadvertently, conductivity and total hardness were not measured at the start of the depuration phase. However, conductivity and total hardness were acceptable at the end of the depuration phase.
14. Water samples for quantitative analysis were taken by use of a mechanical pipette with disposable tips. This had no effect on the outcome of the test.

Evaluation: Deviations 1, 2, 3, 4, 5, 7, 9, 10, 11, 12, 13 and 14 had no effect on the integrity or the final outcome of the study. Deviations 6 and 8 are evaluated in section 7.4.

7. RESULTS

7.1. Measured concentrations:

The results of the radio chemical analyses of samples of the test solution taken during the study are presented in Appendix I. Table 2 summarizes the mean measured concentrations of [U-phenyl-^{14}C] FR-1435X and includes the Time Weighed Average (TWA) concentrations.

The mean TWA concentrations of [U-phenyl-^{14}C] FR-1435X at nominal 0.50 and 5.0 µg/l were 0.52 ± 0.023 µg/l and 4.9 ± 0.16 µg/l, respectively (see also Table 2 for the actual concentrations and the respective calculated TWA concentration per sampling time). The measured concentrations varied within the ± 20% window of the mean TWA concentration at both 0.50 and 5.0 µg/l (see Figure 1 and Figure 2 in Appendix I).

Table 2 Measured [U-phenyl-^{14}C] FR-1435X concentration during the test

Time of measurement (days)	Mean measured [U-phenyl-^{14}C] FR-1435X concentration (µg/l)			
	Target concentration 0.50 µg/l		Target concentration 5.0 µg/l	
	Actual	TWA	Actual	TWA
0	0.49	0.49	4.6	4.6
3	0.52	0.50	5.1	4.8
6	0.50	0.51	4.9	4.9
10	0.53	0.51	5.2	5.0
20	0.59	0.54	5.0	5.0
32	0.56	0.55	4.8	5.0

7.2. Purity of test medium

The results of the purity measurement of the stock and test solutions are presented in Appendix II. The results showed that the purity of [U-phenyl-^{14}C] FR-1435X in the stock solutions was > 97% during the uptake phase.
The low recovery in the 0.50 μg/l sample at the start of the test was the only occasion that purity was substantially below 100% (i.e. 30%). About 60% of the extract remained at the origin of the TLC plate. There was no explanation for this result. The recovery in the 5.0 μg/l sample was 96% at the start of the test. Leaving the 30% recovery out, the purity of [U-phenyl-^{14}C] FR-1435X in the nominal test solutions of 0.50 and 5.0 μg/l were 77 ± 1.4% and 83 ± 7.4% during the main part of the uptake phase, respectively.
After extraction of the water samples with DCM LSC measurements showed that, apart from the samples taken at the start of the test, significant radioactivity remained present in the residual water. This activity was probably due to the presence of one or more metabolites which, would not be effectively extracted in DCM. The fraction of these metabolites remained relatively constant at about 20% of the total activity during the test. The mean percentage of radioactivity found in residual water of the 0.50 and 5.0 µg/l concentrations after extraction with DCM were 20 ± 2.4% and 18 ± 2.1%, respectively.

7.3. Bioconcentration factor

The results of the radio chemical analyses of samples of the fish taken during the study are presented in Appendix III.

Table 3 summarizes the measured concentration of [U-phenyl-^{14}C] FR-1435X in the fish and the bioconcentration factor (BCF) on each sampling point.

During the uptake phase of 32 days the steady state concentration in fish was reached after 6 days of exposure. The mean concentrations of [U-phenyl-^{14}C] FR-1435X in fish at nominal 0.50 and 5.0 μg/l from day 6 until day 32 were 0.40 ± 0.032 μg/g and 4.2 ± 0.43 μg/g, respectively. The measured concentrations varied within the ± 20% window of the mean concentration in fish at both 0.50 and 5.0 µg/l (see Figure 3 and Figure 4 in Appendix III).

The mean steady state BCF of [U-phenyl-^{14}C] FR-1435X at nominal 0.50 and 5.0 μg/l from day 6 until day 32 were 767 ± 44 and 838 ± 88, respectively. The individual BCF's varied within the ± 20% window of the mean BCF at both 0.50 and 5.0 µg/l (see Figure 5 and Figure 6 in Appendix III). Combining the results at the two concentrations tested the BCF_{ss} was 803 ± 75.

The last fish samples were taken on day 41. The study was prolonged to day 46 because on this day the final results of the fish samples taken on day 41 were known: the concentrations in the fish were less than 3% of the concentrations at the start of the depuration phase (2-3%). Further, the curves for depuration showed an exponential decrease of [U-phenyl-^{14}C] FR-1435X in the fish at both levels of exposure (See Figure 3 and Figure 4 in Appendix III). The concentrations found in the fish at the target of 5.0 µg/l were consistently a factor of 10 higher than those found in the tissues at the target of 0.50 µg/l.

Table 3 Measured concentration [U-phenyl-^{14}C] FR-1435X in fish

Time (days)		Nominal concentration: 0.50 µg/l		Nominal concentration: 5.0 µg/l	
		Concentration (µg/ g fish)	BCF	Concentration (µg/ g fish)	BCF
Uptake phase	3	0.33	649	2.8	586
	6	0.38	739	3.9	792
	10	0.41	794	4.6	918
	20	0.39	722	3.7	737
	32	0.45	814	4.5	906
Depuration phase	34	0.13		0.86	
	41	0.012		0.098	

The k_d was determined from the depuration curves. The calculation was performed using the one-compartment model. Both curves decreased exponentially. The slope (k_d) of the curve from the 0.50 and the 5.0 µg/l concentrations were 0.3848 and 0.3955, respectively. Hence, the difference between the two slopes was less than 10%. Further, the first order depuration curves predict well the actual decrease of concentration in the fish with correlation factors of 0.95-0.98. The half-life time (DT_{50}) was 1.8 days for depuration at both target concentrations.

The parameters for the calculation of the BCF_k are presented in Table 4 and Table 5. The mean kinetic BCF's of [U-phenyl-^{14}C] FR-1435X at nominal 0.50 and 5.0 µg/l from day 6 until day 32 were 802 ± 60 and 903 ± 141, respectively. The individual kinetic BCF's varied within the ± 20% window of the mean BCF at 0.50 and was slightly above the ± 20% window (i.e. within the 23% window) of the mean BCF at 5.0 µg/l (see Figure 7 and Figure 8 in Appendix III). Combining the results at the two concentrations tested the BCF_k was 852 ± 114. In addition, the BCF_k was only slightly higher than the BCF_{ss} indicating a rapid depuration.

After 2 days of exposure to the highest test concentration one fish was found dead next to the test vessel and after 36 days (day 5 of depuration) one fish died in the control vessel. These fish were removed when observed. All other fish showed no clinical effects during the entire test period.

Table 4 Parameters for the calculation of the kinetic BCF (nominal 0.5 µg/l)

Exposure time (days)	Tangent (Δc/Δt)	k_d (day^{-1})	C_f (µg/g)	C_w (µg/ml)	Estimated K_u (day^{-1})	BCF_k
3		0.3848	0.33	0.00050		
6	0.016	0.3848	0.38	0.00051	316	822
10	0.008	0.3848	0.41	0.00051	321	833
20	-0.002	0.3848	0.39	0.00054	274	713
32	0.005	0.3848	0.45	0.00055	322	838

Table 5 Parameters for the calculation of the kinetic BCF (nominal 5.0 µg/l)

Exposure time (days)	Tangent (Δc/Δt)	k_d (day^{-1})	C_f (µg/g)	C_w (µg/ml)	Estimated K_u (day^{-1})	BCF_k
3		0.3955	2.8	0.0048		
6	0.352	0.3955	3.9	0.0049	385	974
10	0.166	0.3955	4.6	0.0050	397	1003
20	-0.084	0.3955	3.7	0.0050	275	695
32	0.067	0.3955	4.5	0.0050	372	940

7.4. Experimental conditions

The temperature was measured constantly in all vessels. The average temperatures and their standard deviation are listed in Table 6. At day 31 (uptake phase) temperature was 11.1°C at the 0.50 µg/l concentration, which is below the optimum temperature for Rainbow trout (12-17°C, constant within 1°C). Since this was only for a relatively short period (2 hours maximum) and there were no effects observed on the fish, the study integrity was not adversely affected by this deviation.

Table 6 Temperature of the test solutions

Concentration [U-phenyl-^{14}C] FR-1435X (µg/l)	Average temp. and standard deviation (°C)
control	14.5 ± 0.4
0.50	14.2 ± 0.3
5.0	14.4 ± 0.2

The results of the measurements of pH are presented in Table 7. The pH ranged from 7.5 to 7.9 during the test period, which is within the optimum range for Rainbow trout (6.0-8.5, constant within 1 unit).

Table 7 pH values

Concentration [U-phenyl-^{14}C] FR-1435X (µg/l)	pH								
	Day 0	Day 5	Day 12	Day 19	Day 26	Day 32 uptake	Day 32 depuration	Day 40	Day 46
control	7.7	7.9	7.8	7.8	7.7	7.8	7.8	7.8	7.9
0.50	7.7	7.7	7.5	7.8	7.6	7.8	7.6	7.6	7.8
5.0	7.7	7.8	7.5	7.8	7.6	7.8	7.6	7.7	7.8

The results of the measurements of oxygen concentration are presented in Table 8. Oxygen concentrations varied from 6.2 to 10.5 mg/l during the test period, which is > 60% of saturation as prescribed by the Guideline. Note that aeration was introduced after 1 day of exposure, as the oxygen concentration tended to drop below the optimum level for testing with trout, i.e. below 6 mg/l.

Table 8 Oxygen concentrations

Concentration [U-phenyl-^{14}C] FR-1435X (µg/l)	O_2 (mg/l)									
	Day 0	Day 1[2]	Day 5	Day 12	Day 19	Day 26	Day 32 uptake	Day 32 depuration	Day 40	Day 46
control	10.3/9.9[1]	8.8	10.2	8.2	9.0	9.2	8.3	9.4	9.3	9.4
0.50	10.4	7.5	10.0	6.2[3]	8.4	9.1	8.8	9.7	9.4	9.5
5.0	10.5	7.7	10.3	7.6	8.7	9.4	8.6	9.8	9.1	9.2

[1] First measurement performed at the start, second measurement after 6¼ hours of exposure
[2] Aeration was introduced and maintained until the end of the test
[3] Aeration rate had dropped.

The results of the measurements of conductivity and total hardness are presented in Table 9. Both conductivity and total hardness did not change markedly during the test. Conductivity showed normal levels and total hardness was 10-11 °DH during the test. This corresponds with 179-196 mg calcium carbonate per litre.

Table 9 Conductivity and Total Hardness measurements

Concentration [U-phenyl-^{14}C] FR-1435X (µg/l)	Day 0		Day 32[1]		Day 46	
	C (µs/cm)	H (°DH)	C (µs/cm)	H (°DH)	C (µs/cm)	H (°DH)
control	601	10	612	11	598	10

[1] Measurements were performed at the end of the uptake phase before transition to clean water

The results of the measurements of TOC are presented in Table 10. TOC measurements showed values of 4.5-7.7 mg C/l in samples from the acetone-control during the uptake phase. The major part of this carbon originates from the solvent, which was present at ca. 0.01 ml/l. During the depuration phase TOC was normal at day 41 (1.3 mg/l), but relatively high at day 34 and day 46 (16-17 mg/l). However, these values were measured during the depuration phase and therefore of little relevance on the exposure of the fish to the test substance. The study integrity was not adversely affected by this deviation.

Table 10 TOC measurements

Concentration [U-phenyl-^{14}C] FR-1435X (µg/l)	TOC (mg/l)							
	Day -1	Day 6	Day 13	Day 20	Day 27	Day 34	Day 41	Day 46
control	4.5	7.4	7.1	7.7	7.4	16	1.3	17

8. CONCLUSION

The bioconcentration study investigated the possible bioconcentration of [U-phenyl-^{14}C] FR-1435X in rainbow trout exposed to two different concentrations for a period of 32 days in a flow trough system.

Combining the results at the two concentrations tested the BCF_{ss} was 803 ± 75 and the BCF_k was 852 ± 114. The steady state concentration was reached after 6 days.
The DT_{50} value for depuration of [U-phenyl-^{14}C] FR-1435X was 1.8 days.

Based on these results [U-phenyl-^{14}C] FR-1435X appears to be only slightly accumulating in fish combined with a highly efficient excretion by the fish. Hence there is no substantial risk for bioconcentration of FR-1435X in fish.

APPENDIX I CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN TEST MEDIUM

Nominal day	-1							
Target (µg/l)	**Code**	**Volume (ml)**	**Activity (dpm)**	**Corr. activity (dpm/l)[1]**	**Activity (Bq/l)**	**Concentr. (µg/l)**	**Average (µg/l)**	**% of target**
Control	W10	10	22.15					
	W11	10	25.12					
	W12	10	27.84					
Mean			25.04					
0.50	W13	10	528.42	50338.33	838.97	0.494	0.54	109%
	W14	10	528.43	50339.33	838.99	0.494		
	W15	10	680.69	65565.33	1092.76	0.643		
5.0	W16	10	4549.76	452472.33	7541.21	4.44	4.4	88%
	W17	10	4460.07	443503.33	7391.72	4.35		
	W18	10	4466.87	444183.33	7403.06	4.35		

Nominal day	0							
Target (µg/l)	**Code**	**Volume (ml)**	**Activity (dpm)**	**Corr. activity (dpm/l)[1]**	**Activity (Bq/l)**	**Concentr. µg/l**	**Average µg/l**	**% of target**
Control	W19	10	23.02					
	W20	10	20.52					
	W21	10	19.84					
Mean			21.13					
0.50	W22	10	527.63	50650.33	844.17	0.497	0.49	98%
	W23	10	517.44	49631.33	827.19	0.487		
	W24	10	510.96	48983.33	816.39	0.480		
5.0	W25	10	4718.42	469729.33	7828.82	4.61	4.6	92%
	W26	10	4726.05	470492.33	7841.54	4.61		
	W27	10	4660.44	463931.33	7732.19	4.55		

Nominal day	3							
Target (µg/l)	**Code**	**Volume (ml)**	**Activity (dpm)**	**Corr. activity (dpm/l)[1]**	**Activity (Bq/l)**	**Concentr. (µg/l)**	**Average (µg/l)**	**% of target**
Control	W28	10	29.45					
	W29	10	18.29					
	W30	10	20.78					
Mean			22.84					
0.50	W31	10	546.76	52392.00	873.20	0.514	0.52	104%
	W32	10	558.30	53546.00	892.43	0.525		
	W33	10	555.08	53224.00	887.07	0.522		
5.0	W34	10	5229.99	520715.00	8678.58	5.11	5.1	102%
	W35	10	5241.68	521884.00	8698.07	5.12		
	W36	10	5194.53	517169.00	8619.48	5.07		

APPENDIX I CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN TEST MEDIUM -continued-

Nominal day	6							
Target (µg/l)	Code	Volume (ml)	Activity (dpm)	Corr. activity (dpm/l)[1]	Activity (Bq/l)	Concentr. (µg/l)	Average (µg/l)	% of target
Control	W37	10	20.50					
	W38	10	25.43					
	W39	10	27.23					
Mean			24.39					
0.50	W40	10	551.76	52737.33	878.96	0.517	0.50	101%
	W41	10	513.58	48919.33	815.32	0.480		
	W42	10	550.81	52642.33	877.37	0.516		
5.0	W43	10	4980.44	495605.33	8260.09	4.86	4.9	97%
	W44	10	4902.92	487853.33	8130.89	4.78		
	W45	10	5095.36	507097.33	8451.62	4.97		

Nominal day	10							
Target (µg/l)	Code	Volume (ml)	Activity (dpm)	Corr. activity (dpm/l)[1]	Activity (Bq/l)	Concentr. (µg/l)	Average (µg/l)	% of target
Control	W46	10	20.19					
	W47	10	20.72					
	W48	10	23.70					
Mean			21.54					
0.50	W49	10	573.55	55201.33	920.02	0.541	0.53	107%
	W50	10	574.73	55319.33	921.99	0.542		
	W51	10	552.41	53087.33	884.79	0.520		
5.0	W52	10	5383.83	536229.33	8937.16	5.26	5.2	104%
	W53	10	5346.83	532529.33	8875.49	5.22		
	W54	10	5283.57	526203.33	8770.06	5.16		

Nominal day	20							
Target (µg/l)	Code	Volume (ml)	Activity (dpm)	Corr. activity (dpm/l)[1]	Activity (Bq/l)	Concentr. (µg/l)	Average (µg/l)	% of target
Control	W55	10	20.84					
	W56	10	27.30					
	W57	10	72.43					
Mean			40.19					
0.50	W58	10	651.02	61083.00	1018.05	0.599	0.59	117%
	W59	10	625.40	58521.00	975.35	0.574		
	W60	10	636.48	59629.00	993.82	0.585		
5.0	W61	10	5077.77	503758.00	8395.97	4.94	5.0	100%
	W62	10	5090.88	505069.00	8417.82	4.95		
	W63	10	5271.29	523110.00	8718.50	5.13		

APPENDIX I CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN TEST MEDIUM -continued-

Nominal day	32							
Target (μg/l)	**Code**	**Volume (ml)**	**Activity (dpm)**	**Corr. activity (dpm/l)[1]**	**Activity (Bq/l)**	**Concentr. (μg/l)**	**Average (μg/l)**	**% of target**
Control	W64	10	51.70					
	W65	10	29.79					
	W66	10	23.24					
Mean			34.91					
0.50	W67	10	617.63	58272.00	971.20	0.571	0.56	113%
	W68	10	607.98	57307.00	955.12	0.562		
	W69	10	602.48	56757.00	945.95	0.556		
5.0	W70	10	4026.37	399146.00	6652.43	3.91	4.8	97%
	W71	10	5403.61	536870.00	8947.83	5.26		
	W72	10	5444.62	540971.00	9016.18	5.30		

[1] Corr. activity is the activity measured in water containing test substance minus the mean of the activities measured in the water taken from the control vessel.

APPENDIX I CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN TEST MEDIUM -continued-

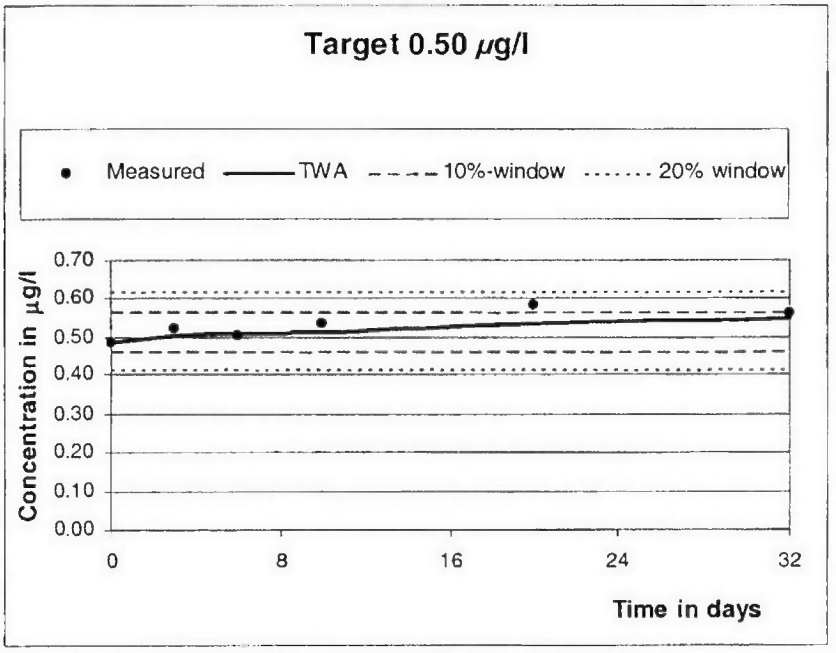

Figure 1 Concentration of [U-phenyl-^{14}C] FR-1435X in test medium (Target concentration in test medium was 0.50 µg/l)

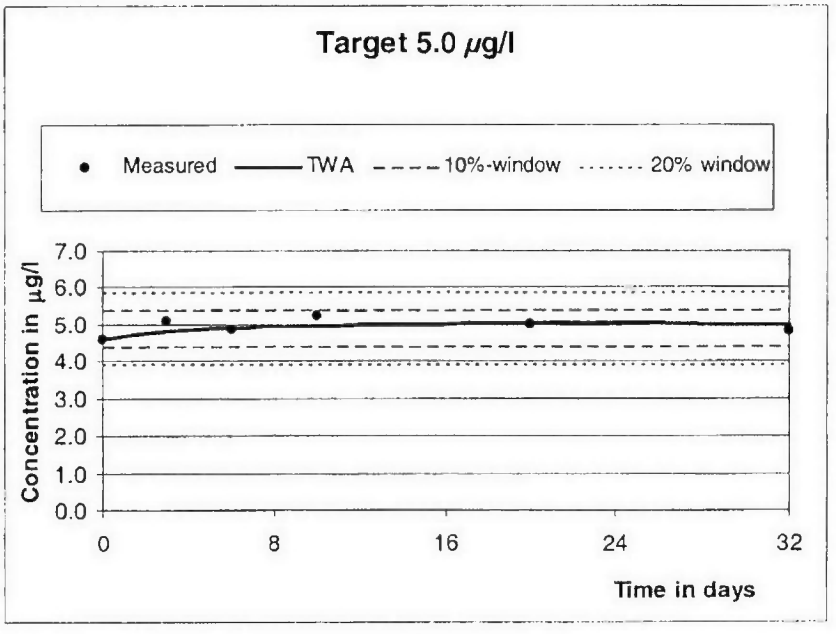

Figure 2 Concentration of [U-phenyl-^{14}C] FR-1435X in test medium (Target concentration in test medium was 5.0 µg/l)

APPENDIX II PURITY OF TEST MEDIUM

Table 11 **Results of Thin Layer Chromatography (TLC) analysis on purity and stability of [U-phenyl-^{14}C] FR-1435X in stock and test solutions. DCM is dichloromethane, LSC is Liquid scintillation.**

Sample	Type	Conc.	Day	LSC			TLC	
				DCM extract (%)	Residual water extract (%)	Total recovery (%)#	DCM extract on plate (%)	Purity (%)
stC	Stock	50 mg/l	0				99.3	99.3
stB	Stock	500 mg/l	0				98.8	98.8
1	Test sol.	0.50 µg/l	0	116.0	0	116.0	30	30.0 †
2	Test sol.	5.0 µg/l	0	138.5	0.3	138.8	96.6	96.4 †
stC	Stock	50 mg/l	7				99.1	99.1
stB	Stock	500 mg/l	7				98.8	98.8
3	Test sol.	0.50 µg/l	7	94.6	16.8	111.4	92.4	78.5 †
4	Test sol.	5.0 µg/l	7	106.4	16.8	123.2	95.4	82.4 †
stC	Stock	50 mg/l	14				100	100
5	Test sol.	0.50 µg/l	14	91.6	22.7	114.2	96.5	77.4 †
6	Test sol.	5.0 µg/l	14	93.1	20.9	114.1	96.9	79.1 †
stC	Stock	50 mg/l	21				97.1	97.1
7	Test sol.	0.50 µg/l	21	103.9	20.3	124.2	90.2	75.5 †
8	Test sol.	5.0 µg/l	21	102.2	16.3	118.5	91.7	79.1 †
stC	Stock	50 mg/l	27				98.1	98.1
9	Test sol.	0.50 µg/l	27	81.3	19.9	101.2	94.4	75.8 †
10	Test sol.	5.0 µg/l	27	99.8	17.4	117.2	94.3	80.3 †

Percentage of the total radioactivity measured in the original water sample.
† Purity corrected for the relative fraction present in the DCM extract against the total recovery.

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH

Nominal day	3								
Target (µg/l)	**Code**	**Weighed amount(mg)**	**Activity (dpm)**	**Corr. activity (dpm/g)[1]**	**Activity Bq/g**	**Conc. µg/g fish**	**Average µg/g fish**	**TWA (µg/ml)[2]**	**BCF**
Control	F1,2a	200.25	371.55						
	F1,2b	199.66	354.85						
	F1,2c	199.82	368.23						
Mean			364.88						
0.50 A	F3,4a	200.56	7226.93	34213.77	570.23	0.335	0.33	5.0E-04	661
	F3,4b	200.08	7124.87	33786.95	563.12	0.331			
	F3,4c	200.02	7142.72	33886.47	564.77	0.332			
0.50 B	F5,6a	199.89	6933.92	32863.06	547.72	0.322	0.32	5.0E-04	638
	F5,6b	199.99	6923.34	32793.93	546.57	0.322			
	F5,6c	199.63	6879.46	32632.51	543.88	0.320			
5.0 A	F7,8a	200.00	50862.40	252492.83	4208.21	2.48	2.5	4.8E-03	512
	F7,8b	199.36	50589.00	251930.18	4198.84	2.47			
	F7,8c	199.18	50711.40	252773.55	4212.89	2.48			
5.0 A	F7,8d	196.98	50319.80	253598.87	4226.65	2.49	2.5	4.8E-03	513
	F7,8e	199.99	50971.40	253051.31	4217.52	2.48			
	F7,8f	197.91	50474.90	253196.12	4219.94	2.48			
5.0 B	F9,10a	200.23	65526.40	325437.83	5423.96	3.19	3.2	4.8E-03	661
	F9,10b	200.32	65846.90	326890.32	5448.17	3.20			
	F9,10c	200.31	65607.50	325711.62	5428.53	3.19			

Nominal day	6								
Target (µg/l)	**Code**	**Weighed amount(mg)**	**Activity (dpm)**	**Corr. activity (dpm/g)[1]**	**Activity Bq/g**	**Conc. µg/g fish**	**Average µg/g fish**	**TWA (µg/ml)[2]**	**BCF**
Control	F11,12a	198.78	2.23						
	F11,12b	200.35	1.34						
	F11,12c	200.59	3.57						
Mean			2.38						
0.50 A	F13,14a	196.12	7617.89	38831.81	647.20	0.381	0.38	5.1E-04	751
	F13,14b	195.96	7615.21	38848.71	647.48	0.381			
	F13,14c	197.38	7718.83	39094.31	651.57	0.383			
0.50 B	F15,16a	200.02	7516.59	37567.55	626.13	0.368	0.37	5.1E-04	727
	F15,16b	196.91	7465.11	37899.10	631.65	0.372			
	F15,16c	199.00	7481.21	37581.48	626.36	0.368			
5.0 A	F17,18a	199.65	79715.00	399258.43	6654.31	3.91	3.9	4.9E-03	799
	F17,18b	198.85	79581.70	400189.66	6669.83	3.92			
	F17,18c	198.89	79619.20	400296.94	6671.62	3.92			
5.0 B	F19,20a	198.86	78365.80	394054.47	6567.57	3.86	3.9	4.9E-03	786
	F19,20b	200.22	78759.30	393359.53	6555.99	3.86			
	F19,20c	200.50	78960.80	393813.75	6563.56	3.86			

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

Nominal day	10								
Target (µg/l)	**Code**	**Weighed amount(mg)**	**Activity (dpm)**	**Corr. activity (dpm/g)[1]**	**Activity Bq/g**	**Conc. µg/g fish**	**Average µg/g fish**	**TWA (µg/ml)[2]**	**BCF**
Control	F21,22a	200.29	18.23						
	F21,22b	200.09	5.25						
	F21,22c	199.97	11.61						
Mean			11.70						
0.50 A	F23,24a	199.99	7930.08	39593.61	659.89	0.388	0.39	5.1E-04	756
	F23,24b	200.18	7873.93	39275.21	654.59	0.385			
	F23,24c	200.32	7950.13	39627.91	660.47	0.389			
0.50 B	F25,26a	200.01	8693.25	43406.07	723.43	0.426	0.43	5.1E-04	832
	F25,26b	199.68	8693.66	43480.05	724.67	0.426			
	F25,26c	200.09	8729.59	43570.29	726.17	0.427			
5.0 A	F27,28a	199.70	91287.10	457066.27	7617.77	4.48	4.5	5.0E-03	906
	F27,28b	199.75	91496.50	458002.78	7633.38	4.49			
	F27,28c	199.84	92015.50	460375.82	7672.93	4.51			
5.0 B	F29,30a	200.03	94332.80	471527.33	7858.79	4.62	4.6	5.0E-03	931
	F29,30b	200.43	94224.80	470058.98	7834.32	4.61			
	F29,30c	200.42	94436.20	471138.16	7852.30	4.62			

Nominal day	20								
Target (µg/l)	**Code**	**Weighed amount(mg)**	**Activity (dpm)**	**Corr. activity (dpm/g)[1]**	**Activity Bq/g**	**Conc. µg/g fish**	**Average µg/g fish**	**TWA (µg/ml)[2]**	**BCF**
Control	F31,32a	200.81	11.56						
	F31,32b	199.74	9.76						
	F31,32c	200.28	10.88						
Mean			10.73						
0.50 A	F33,34a	199.72	7702.25	38512.33	641.87	0.378	0.38	5.4E-04	706
	F33,34b	199.39	7744.35	38786.22	646.44	0.380			
	F33,34c	199.72	7704.50	38523.60	642.06	0.378			
0.50 B	F35,36a	199.89	8121.78	40577.31	676.29	0.398	0.40	5.4E-04	738
	F35,36b	200.04	8056.52	40220.73	670.35	0.394			
	F35,36c	199.89	8069.06	40313.56	671.89	0.395			
5.0 A	F37,38a	200.12	72577.10	362617.69	6043.63	3.56	3.6	5.0E-03	706
	F37,38b	199.98	72626.20	363118.30	6051.97	3.56			
	F37,38c	200.73	72809.60	362668.70	6044.48	3.56			
5.0 B	F39,40a	199.64	78649.90	393902.78	6565.05	3.86	3.9	5.0E-03	768
	F39,40b	199.87	78708.20	393751.24	6562.52	3.86			
	F39,40c	199.28	78956.50	396152.57	6602.54	3.88			

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

Nominal day	32								
Target (µg/l)	Code	Weighed amount(mg)	Activity (dpm)	Corr. activity (dpm/g)[1]	Activity Bq/g	Conc. µg/g fish	Average µg/g fish	TWA (µg/ml)[2]	BCF
Control	F41,42a	200.17	2.67						
	F41,42b	200.49	0						
	F41,42c	200.39	0						
Mean			0.89						
0.50 A	F43,44a	200.14	8341.67	41675.06	694.58	0.409	0.41	5.4E-04	766
	F43,44b	199.81	8457.86	42324.98	705.42	0.415			
	F43,44c	199.88	8372.60	41883.69	698.06	0.411			
0.50 B	F45,46a	199.27	9846.87	49410.71	823.51	0.484	0.48	5.4E-04	903
	F45,46b	199.27	9865.11	49502.25	825.04	0.485			
	F45,46c	198.57	9812.12	49409.98	823.50	0.484			
5.0 A	F47,48a	199.97	89034.50	445238.81	7420.65	4.37	4.4	4.9E-03	892
	F47,48b	199.28	88676.90	444979.67	7416.33	4.36			
	F47,48c	200.06	88878.90	444261.56	7404.36	4.36			
5.0 B	F49,50a	200.19	95853.50	478813.03	7980.22	4.69	4.7	4.9E-03	957
	F49,50b	200.21	95285.70	475929.70	7932.16	4.67			
	F49,50c	200.06	95413.00	476918.66	7948.64	4.68			

Nominal day	34						
Target (µg/l)	Code	Weighed amount(mg)	Activity (dpm)	Corr. activity (dpm/g)[1]	Activity Bq/g	Conc. µg/g fish	Average µg/g fish
Control	F51,52a	199.940	5.71				
	F51,52b	199.908	4.58				
	F51,52c	199.612	6.07				
Mean			5.45				
0.50 A	F53,54a	199.425	2706.62	13544.80	225.75	0.13	0.13
	F53,54b	199.848	2765.96	13813.03	230.22	0.14	
	F53,54c	199.383	2725.11	13640.34	227.34	0.13	
0.50 B	F55,56a	200.123	2516.01	12545.06	209.08	0.12	0.12
	F55,56b	199.932	2505.28	12503.37	208.39	0.12	
	F55,56c	200.224	2503.29	12475.24	207.92	0.12	
5.0 A	F57,58a	199.531	18020.6	90287.61	1504.79	0.89	0.88
	F57,58b	199.968	18011.6	90045.26	1500.75	0.88	
	F57,58c	199.419	18003.7	90253.49	1504.22	0.88	
5.0 B	F59,60a	199.734	17168.2	85927.82	1432.13	0.84	0.84
	F59,60b	199.594	17200.7	86151.26	1435.85	0.84	
	F59,60c	199.574	17226.8	86290.73	1438.18	0.85	

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

Nominal day	41						
Target (µg/l)	**Code**	**Weighed amount(mg)**	**Activity (dpm)**	**Corr. activity (dpm/g)[1]**	**Activity Bq/g**	**Conc. µg/g fish**	**Average µg/g fish**
Control	F61,62a	197.340	0				
	F61,62b	200.244	0				
	F61,62c	200.476	2.6				
Mean			0.87				
0.50 A	F63,64a	199.382	251.97	1259.41	20.99	0.012	0.012
	F63,64b	199.888	249.49	1243.81	20.73	0.012	
	F63,64c	200.313	253.35	1260.44	21.01	0.012	
0.50 B	F65,66a	201.662	242.12	1196.33	19.94	0.012	0.012
	F65,66b	201.509	251.35	1243.04	20.72	0.012	
	F65,66c	200.999	239.39	1186.69	19.78	0.012	
5.0 A	F67,68a	198.966	2279.18	11450.76	190.85	0.11	0.11
	F67,68b	199.504	2267.17	11359.66	189.33	0.11	
	F67,68c	199.432	2272.85	11392.27	189.87	0.11	
5.0 B	F69,70a	199.251	1708.99	8572.74	142.88	0.08	0.08
	F69,70b	200.022	1751.95	8754.43	145.91	0.09	
	F69,70c	199.488	1710.81	8571.66	142.86	0.08	

[1] Corr. activity is the activity measured in fish exposed to the test substance minus the mean of the activities measured in fish from the control vessel.
[2] TWA is the Time Weighed Average concentration in water.

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

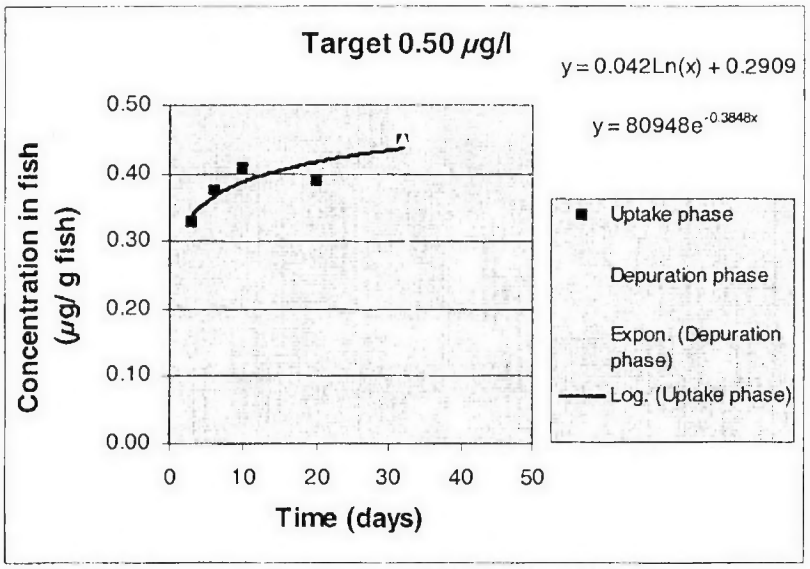

Figure 3 Concentration of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 0.50 µg/l)

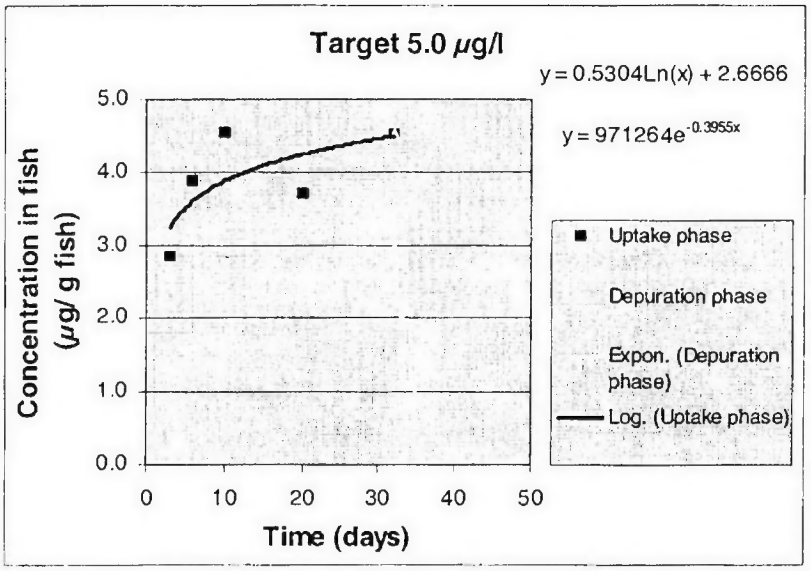

Figure 4 Concentration of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 5.0 µg/l)

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

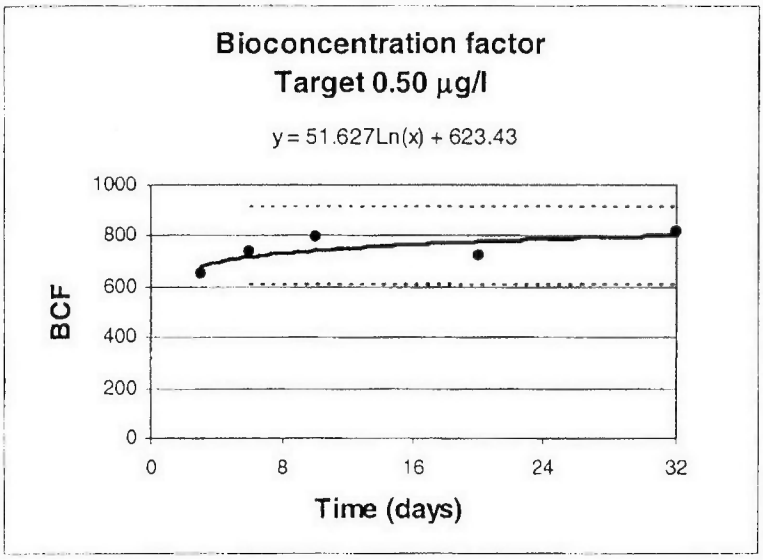

Figure 5 Bioconcentration factor of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 0.50 µg/l)

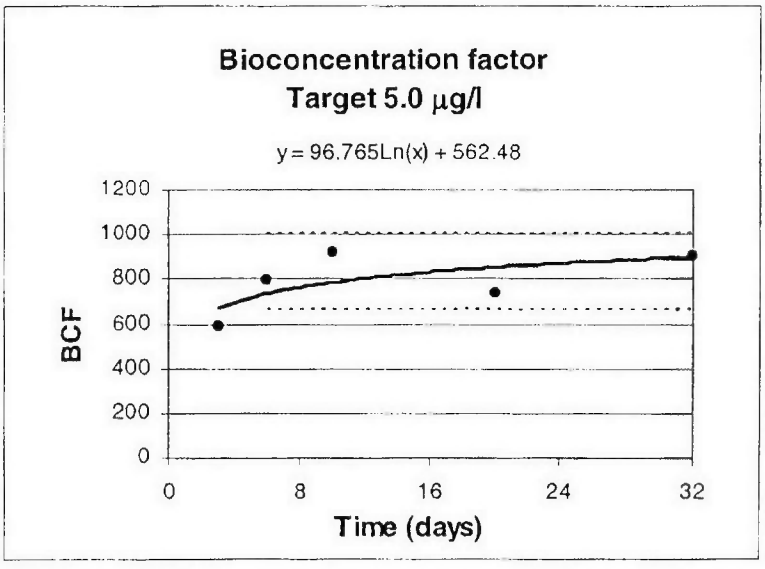

Figure 6 Bioconcentration factor of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 5.0 µg/l)

APPENDIX III CONCENTRATION OF [U-phenyl-^{14}C] FR-1435X IN FISH -continued-

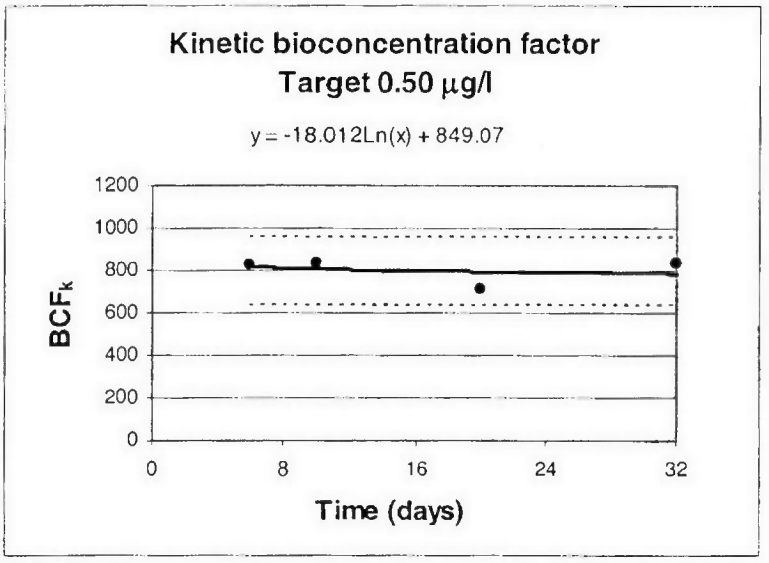

Figure 7 Kinetic bioconcentration factor of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 0.50 µg/l)

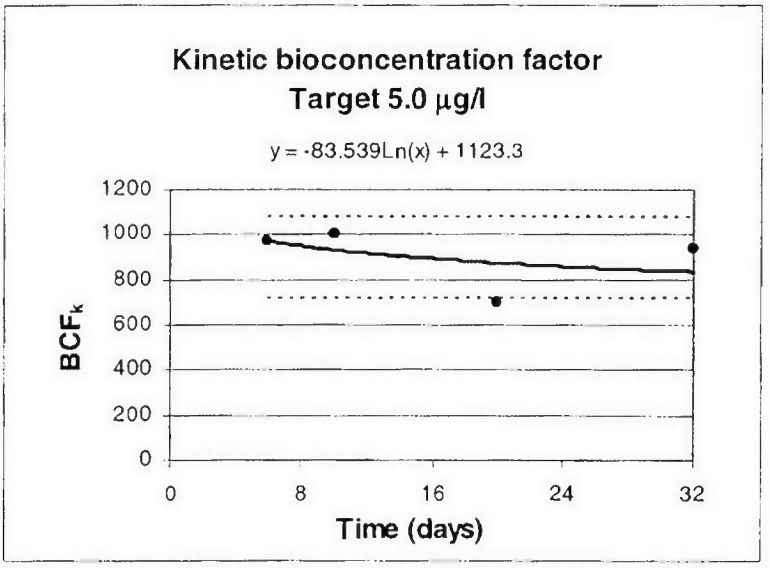

Figure 8 Kinetic bioconcentration factor of [U-phenyl-^{14}C] FR-1435X in fish (Target concentration in test medium was 5.0 µg/l)

APPENDIX IV ANALYSIS OF TAP WATER (Not performed under GLP)

Date of sampling : 08-06-05
Sample numbers : 2106017
Laboratory : ANALYTICO MILIEU B.V., Barneveld, The Netherlands

COMPONENT:	ANALYSIS:	
Metals:		
Selenium	<0.90	µg/l
Arsenic	<5.0	µg/l
Cadmium	<0.40	µg/l
Chromium	<1.0	µg/l
Copper	29	µg/l [a)]
Mercury	<0.050	µg/l
Lead	<5.0	µg/l
Zinc	19	µg/l
Manganese	<0.010	mg/l
Calcium	53	mg/l [a)]
Magnesium	5.2	mg/l [a)]
Iron	<0.050	mg/l
EOX (extractable organic halogens)	<1.0	µg/l
Polycyclic Aromatic Hydrocarbons:		
Naphtalene	<0.010	µg/l
Acenaphtylene	<0.050	µg/l
Acenaphtene	<0.010	µg/l
Fluorene	<0.010	µg/l
Phenanthrene	<0.010	µg/l
Anthracene	<0.0050	µg/l
Fluoranthene	<0.010	µg/l
Pyrene	<0.010	µg/l
Benzo(a)antracene	<0.010	µg/l
Chrysene	<0.010	µg/l
Benzo(b)fluoranthene	<0.010	µg/l
Benzo(k)fluoranthene	<0.010	µg/l
Benzo(a)pyrene	<0.010	µg/l
Dibenzo(ah)antracene	<0.010	µg/l
Benzo(ghi)perylene	<0.010	µg/l
Indeno(123cd)pyrene	<0.010	µg/l
PAC's Total EPA (16)		
PAC's Total VROM (10)		

Total aerobic germ-count (22ºC)	42	CFU/ml [a)]
Total aerobic germ-count (37ºC)	<1	CFU/ml [a)]
Hardness (expr. as Ca + Mg):	1.6	mmol/l [a)]
Nitrate (expr. as N):	0.67	mg/l
Nitrite (expr. as N):	0.018	mg/l
Cyanide-totaal (NEN 6655)	<1.0	ug/l
Troebelheid	0.049	FTE
Kleurintensiteit	20	mg Pt/l

Dechlorination of tap water was not necessary since no chlorine was present.

[a)] Tap water was sampled from the copper-free water supply system that supplied the water used for the present toxicity study. Among others, copper and bacterial activity were measured in these samples. The other components were analysed in samples taken from the same water supply but sampled in the Animal House.

APPENDIX V INGREDIENTS OF Nutra (Not performed under GLP)

The following data originate from Trouw Nutrition B.V., Putten, The Netherlands.

Rough protein	55 %
Rough fat	16 %
Cellulose	1 %
Rough ashes	12 %
Carbohydrates	8 %
Phosphor	1.6 %
Vitamine A	10,000 IE/kg
Vitamine D3	1,500 IE/kg
Vitamine E	250 IE/kg

APPENDIX VI CERTIFICATE OF ANALYSIS

Caution-Radioactive material

SCYNEXIS

Certificate of Analysis

SEL/ 1489A

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Chemical name :

Common name:

Compound number: FR-1435X

Sample supplied as: Colourless oil (109.2mg, 185.6MBq, 5.02mCi and 115.7mg, 196.7MBq, 5.32mCi) in screw-capped glass vials

Recommended storage conditions: Glass bottle < -15°C

Sample identity

Batch number: SEL/1489A

Appearance: Colourless oil

Location of synthesis: Radiochemistry, Ongar

Month of production: January-05

Analytical and other data

Method of determination:	Radiopurity	Date
HPLC (Acetonitrile:water, 75% to 100% acetonitrile over 20min)	97.2%	14-Jan-05
TLC (Toluene/MTBE 40:1) and autoradiography	99.2%	17-Jan-05

Chemical Purity determined by: ^{1}H - NMR: ~99 % HPLC: 98.0% @ 230nm

Specific activity determined by gravimetric analysis:	1077.4 MBq/mmol	29.12 mCi/mmol
	1.70 MBq/mg	45.95 µCi/mg

Characterisation: ^{1}H-NMR, LC-MS, TLC & HPLC compared to Certified Standard Ref: FR-1435X, Batch 38278-53-4

We certify that this data is an accurate summary of the analysis data held at Scynexis *Europe Limited*.
All data has been obtained following internal Quality Control Procedures.

Radiochemist: D Rustidge QC Officer: R Burton Date

20 JAN 2005

Caution: For research use only. Not intended or recommended for use externally or internally in humans

SCYNEXIS Europe Limited, Fyfield Business and Research Park, Fyfield Road, Ongar, Essex, CM5 0GS, United Kingdom.
Registered Office: Hale and Dorr, Alder Castle, 10 Noble Street, London, EC2V 7QJ, United Kingdom
www.scynexis.com

- Attachment 19 -

ICL Industrial PRODUCTS
IMI TAMI Institute for Research and Development LTD
P.O.Box 10140 Haifa Bay 26111 www.tami-imi.com
Tel: 972-4-8469485 Fax: 972-4-8469344 shani@tami-imi.icl-ip.com

Date: 16.10.2006

Identification of FR-1435X by FT-IR spectroscopy

General

CONFIDENTIAL

The sample was prepared as KBr pellet (0.72 mg of FR-1435/ 71 mg of KBr).

Conditions:

- transmittance mode;
- spectral range: 4400-400 cm^{-1};
- 10 scans;
- resolution: 4 cm^{-1};
- interval: 4 cm^{-1};

Results

The FT-IR spectrum of FR-1435X is attached. The interpretation of the major bands is as follow:

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Tel: 972-4-8469485 Fax: 972-4-8469344 shani@tami-imi.icl-ip.com

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Performed by: Dr. S. Sheffer Dee-Noor

QC (Pharma) laboratory

Tami (IMI) Institute for Research and Development Ltd.

Haifa, Israel

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

APR 2 4 2009

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

Dr. Andy Wang
Regulatory Affairs Manager
ICL-IP America, Inc.
420 Saw Mill River Road
Ardsley, NY 10502

[redacted]

Re: PMN P-09-0135

Dear Dr. Wang:

This letter concerns the above-referenced premanufacture notice ("PMN") which you submitted pursuant to section 5(a) of the Toxic Substances Control Act ("TSCA") and 40 CFR Part 720. The PMN described the chemical substance as FR[redacted]

The Environmental Protection Agency ("EPA") has determined, under section 5(e) of TSCA, that available information is insufficient to permit a reasoned evaluation of the potential environmental and human health effects of the PMN substance. Further, EPA has determined, that the manufacturing, processing, distribution in commerce, use, or disposal of the substance may present an unreasonable risk to human health and the environment. This letter provides you with the basis of EPA's determinations and describes your options in light of these determinations.

Based on the physical/chemical properties of the PMN substance and test data on structurally similar substances, the PMN substance is a potentially persistent, bioaccumulative, and toxic ("PBT")[1] chemical. EPA estimates that the PMN substance will persist in the environment more than 2 months and estimates a bioaccumulation factor of greater than or equal to 1,000. During review of the PMN substance, the Agency identified the following health concerns with the PMN substance: Concern for dermal sensitization based on test data on the PMN substance; concern for liver toxicity and neurotoxicity based on test data on [redacted] compounds; concern for mutagenicity based on test data on an analog that had positive results for sister chromatid exchange and in a mouse lymphoma assay; concern for developmental toxicity based on 8(e) test data; and concern for oncogenicity based on[redacted]

[1] See www.epa.gov/opptintr/newchems/pubs/chemcat.htm, www.epa.gov/opptintr/newchems/pbtpolicy.htm, and 64 FR 60914, Nov. 4, 1999.

In addition, based on acute test data on the PMN substance, EPA expects chronic toxicity to aquatic organisms to occur at a concentration of 6 parts per billion of the PMN substance in surface waters. EPA expects releases of this PMN substance to water to result in surface water concentrations significantly exceeding that concern level. During use operations, surface water concentrations are estimated to reach 23 parts per billion, and exceed the chronic COC for 67 days per year.

Given EPA's determinations under section 5(e), EPA will regulate this substance pending the development of sufficient information. Your company has three options available.

Option 1. Your first option is, before commencing any manufacture or import of the PMN substance, to develop and submit data according to the testing scheme charted below. The potential for persistence (P), bioaccumulation (B) and toxicity (T) are each weighed in the Agency's risk assessment. The testing strategy outlined below is based on the November 4, 1999 PBT policy statement and is intended to establish or refute the PBT potentials of the PMN substance. The first tier of tests addresses persistence, the second tier addresses bioaccumulation, and the third tier focuses on toxicity, but includes testing to address any remaining environmental fate issues. A chemical's PBT classification is determined from a combination of these three testing tiers.

Once a chemical becomes distributed in the environment at low concentrations, the properties of persistence and bioconcentration can result in residues high enough to approach a toxic dose in organisms. The first two testing tiers focus on P and B because of the critical role these aspects play in PBT determinations and because of their lower cost. Thus, chronic toxicity testing, which is expected to be the most expensive testing, is reserved until Tier 3 where it serves to determine whether a chemical meets EPA's PBT criteria for new chemicals. Although the early tier P and B testing may either obviate the need for toxicity testing or result in more directed and cost-effective toxicity testing, the need for toxicity testing can be considered in each testing tier.

Test data on the PMN substance found that the measured Log Octanol-Water Partition Coefficient (Log K_{ow}) of the PMN substance is 5.6. Because this value is >= 4.2, the testing strategy below starts with Tier 2. After the second tier of testing is submitted, EPA will review the data and determine the appropriate regulatory action. If the testing indicates a continued concern for persistence, bioaccumulation and toxicity, EPA may request you to conduct further testing identified below in Tier 3. Other health testing may also be considered where appropriate.

Tier 2. **Biodegradability and Bioaccumulation**

- Fish bioconcentration factor (BCF) test BCF (OPPTS 850.1730/OECD 305). The measured BCF should be based on 100 percent active ingredient and measured concentration(s).

Tier 3. **Toxicity/advanced environmental fate testing.**

- Combined repeated dose oral toxicity with the reproductive/developmental toxicity screening test (OPPTS 870.3650, OECD No. 422) in rats.
- Fish early-life stage toxicity test (OPPTS 850.1400/OECD 210) conducted using the continuous renewal method due to the low solubility of the PMN substance.
- Daphnid chronic toxicity test (OPPTS 850.1300/OECD 202) conducted using the continuous renewal method.

EPA strongly encourages you, before performing any testing, to consult with the Agency pertaining to protocol selection. Many test guidelines are now available on the Internet at www.epa.gov/opptsfrs/home/guidelin.htm. Although the guidelines provide general guidance for development of test protocols, they are not themselves acceptable protocols. Test data should also be developed according to TSCA Good Laboratory Practice Standards ("GLPS") at 40 CFR Part 792 and through the use of methodologies generally accepted at the time the study is initiated. Failure to obtain protocol approval or follow GLPS could result in data insufficient to permit a reasoned evaluation of the effects of the substance. Any test data submitted should include a certificate of analysis, protocols, raw data, and results.

To pursue the first option, you must agree to suspend the PMN review period for a time period sufficient to permit development and review of the additional information or test data. Suspensions of the PMN review period are authorized by 40 CFR 720.75(b).

Option 2. A second option is, before commencing any manufacture or import of the PMN substance may commence, to submit to EPA additional information on the potential effects, releases, or exposures of the PMN substance that you believe may refute EPA's finding of potential unreasonable risk under section 5(e) of TSCA as described above. Upon Agency review, such information may influence EPA's determination of whether and how to regulate this chemical substance and might substitute for some or all of the testing described in Option 1. A suspension of the review period for a time adequate to allow for EPA to review the submitted information may also be necessary for Option 2.

The Agency strongly encourages you to consider exploration of methods of source reduction, pollution prevention, or recycling. The Pollution Prevention Act of 1990 and EPA's Pollution Prevention Strategy (56 Federal Register 7849, February 26, 1991), rank preferences in methods of controlling chemical risks as follows: source reduction first, recycling second, treatment third, and disposal last. The rationale for this ranking order is that, environmentally and economically, it is usually better to avoid creation of a pollutant than to subsequently control exposures and releases by shifting a pollutant among environmental media (e.g., water, air, land). For additional information on pollution prevention, you may view EPA's Pollution Prevention Home-Page on the Internet at www.epa.gov/oppt/p2home and utilize the Pollution Prevention Information Clearinghouse at www.epa.gov/oppt/ppic, e-mail: ppic@epa.gov, (202) 566-0799.

Option 3. A third option is to withdraw your PMN. Such a withdrawal will not prejudice any right to resubmit in the future a PMN or exemption notice for the same substance

to EPA under section 5(a) or 5(h) of TSCA. A written notice of withdrawal must be sent to EPA in accordance with 40 CFR 720.75(e).

Response to EPA in 30 days. Within 30 days of your receipt of this letter, please complete and return to EPA's Program Manager the enclosed "Selection of Regulatory Options" form, which will notify EPA of your company's decision as to which option it wishes to pursue. If you decide to pursue certain options, you should also include a suspension of the PMN review period on the enclosed form, as indicated there.

Failure to respond. If you do not complete and return, within 30 days, the enclosed form indicating your decision and providing an adequate suspension of the review period to pursue one of the above options, EPA will assume that you do not wish to pursue a negotiated approach to the resolution of this PMN review. Under this scenario, EPA may unilaterally issue a proposed Order under section 5(e) of TSCA that may completely prohibit the manufacture, processing, distribution in commerce, use, or disposal of the PMN substance pending the development of information sufficient to permit a reasoned evaluation of the effects of the substance. Consequently, my staff would issue a notice under section 5(c) of TSCA extending the PMN review period to 180 days to permit development of the Order under section 5(e). In addition, you would be contacted by a representative of the Information Management Division ("IMD"), Office of Pollution Prevention and Toxics ("OPPT"), and required to substantiate all Confidential Business Information ("CBI") claims in your PMN as a condition of maintaining the information as CBI.

Under the procedures in section 5(e), EPA would issue the proposed Order no later than day-135 of the PMN review period and, on or before the day the Order is issued, notify you in writing of the substance of the determinations underlying the Order. The Order would become effective upon the expiration of the review period, unless you file with EPA, within 30 days of receiving the notification, formal objections to the Order specifying with particularity the provisions of the Order deemed objectionable and stating the grounds therefore. EPA would review the objections and decide whether to apply to a United States District Court for an injunction to enforce the terms of the Order. This process is described fully in section 5(e) of TSCA.

EPA contact. If you have any questions or comments, please contact Karen Chu, the Program Manager for this PMN, at (202) 564-8773.

Sincerely,

for

Greg Schweer, Chief
New Chemicals Notice Management Branch
Chemical Control Division (7405 M)

Enclosure

[redacted]

SELECTION OF REGULATORY OPTION

FOR PMN P-09-135

I have reviewed the Agency's letter dated _____, which outlines the regulatory options for the above-referenced PMN, and I select the following option:

☐ Option 1 - Upfront submission of before commencement of specified data before commencement of manufacture
(suspension as appropriate)

☐ Option 2 - Upfront submission of additional information before commencement of manufacture
(suspension as appropriate)

☐ Option 3 - Withdrawal of PMN per 40 CFR 720.75(e)
(no suspension necessary)

To provide time for implementation of the selected option, I request, pursuant to 40 CFR 720.75(b), a suspension of the PMN review period until the following date: _____.

_____ _____

Date Signature

Name:

Title:

Company: ICL-IP America, Inc.

TSCA CONFIDENTIAL BUSINESS INFORMATION DOES NOT CONTAIN NATIONAL SECURITY INFORMATION (E.O. 12065)

TSCA CONFIDENTIAL BUSINESS INFORMATION DOES NOT CONTAIN NATIONAL SECURITY INFORMATION (E.O. 12065)

Focus Report

New Chemicals Program

PMN Number: **P-09-0135**

Focus Date:	01/15/2009 12:00:00 AM	Report Status:	Completed
Consolidated Set:			
Focus Chair:	Miriam Wiggins-Lewis	Contractor:	Christina Stanley

I. Notice Information

Submitter: ICL-IP America Inc.

CAS Number: [redacted]

Chemical Name: [redacted]

Use: Flame retardant for polyurethane foam. [redacted] it is for use as a flame retardant for polymers. Analog [redacted] is a flame retardant for polyurethane foams.

Other Uses: [redacted]

PV-Max: [redacted] Kg/yr

Manufacture: Import: X

II. SAT Results

(1) **Health Rating:** 1-2 **Eco Rating:** 3 **Comments:** ;chronic only

Occupational: 1B **Non-Occupational:** 1 **Environmental:** 2

(1) **PBT:** 2 2 2 **Comments:**

III. OTHER FACTORS

Categories:

Health Chemical Category: Ecotox Category:

Related Cases/Regulatory History:

Health related Cases:

Ecotox Related Cases: Analogs: [redacted].

Regulatory History:

MSDS/Label Information:

MSDS: Yes Label: No

General Equipment: protective gloves / chemical safety goggles / body covering and boots / provide adequate ventilation.

Respirator: approved respirator

Health Effects: mild eye irritant

TLV/PEL (PMN or raw material): - none established

[redacted]

[redacted] Summary of SAT Assessment

Fate:

Fate Summary: P-09-0135

FATE:

[redacted]

[redacted]
BP = Dec. >220 EC (M)
H < 1.00E-8 (E)
POTW removal (%) = 90 via sorption and possible partial biodeg; OECD 310 (CO2 in sealed vessels): NRB/28 d.
Time for complete ultimate aerobic biodeg = mo
Sorption to soils/sediments = strong - v.strong
PBT Potential: P2B2
*CEB FATE: Migration to ground water = negl

Health:

Health Summary: Expect poor absorption via all routes (pchem). Concern for dermal sensitization based on submitted test data. Concern for liver toxicity and neurotoxicity based on brominated compounds. Concern for mutagenicity [(+) for sister chromatic exchange, (+) in a mouse lymphoma assay]; uncertain concern for developmental toxicity [[redacted], LOEL = 160 mg/kg] and marginal concern for oncogenicity ([redacted]) based on [redacted].

Test Data: (-) Salmonella with and without activation; (-) E. coli with and without activation; rat dermal LD0 = 2000 mg/kg; slight skin irritation in rabbits, cleared by 48 - 72 hours; mild eye irritation in rabbits; (+) for skin sensitization in a mouse local lymph node assay, EC3 = 23%

Ecotox:

Ecotox Values:

Fish 96-h LC50:	*(P)	0.76(M)
Daphnid 48-h LC50:	*(P)	0.64(M)
Green algal 96-h EC50:	*(P)	0.56(M)
Fish Chronic Value:	0.007(P)	
Daphnid ChV:	0.014(P)	
Algal ChV:	0.016(P)	

Ecotox values comments: Predictions are based on SARs for neutral organic chemicals; SAR chemical class = h[redacted], MW[redacted]

This case had ecotoxicity studiies. See the attached spreadsheet for evaluation and details. Conclusions are provided below.

Conclusion: Results from the three tests are considered valid. Due to low water solubility, low nominal concentrations were used that generally agreed with measured concentrations in the fish and daphnia tests. Because measured concentrations decreased over time, flow-through, continuous renewal was emplyed for the fish and daphnia tests. For the static algae test, continuous renewal was not possible and measured concentrations dropped over time. Algae appear to be the most sensitive species with a 72-hour EC50 of 0.56 mg/L (for biomass). The concentration of concern (CC) may be derived by dividing this value by 10 to simulate a chronic EC50, then dividing again by an adjustment (uncertainty) factor of 10, yeilding a CC of 0.0056 mg/L or 5.6 ug/L, rounded to 6 ppb.

Ecotox Factors:

Assessment Factor:	10
Concern Concentration:	6

[redacted]

VI. Focus Decision and Rationale

Regulatory Actions

Regulatory Decision: PMN Ban Pending Upfront Testing Decision Date: 01/15/2009
Type of Decision:

Rationale: P09-0135 will be regulated under the TSCA 5(e) category for neutral organics under the risk based authority for eco and fate. This PMN substance will also be regulated for PBT health concerns. Potential health concerns resulted from the PBT rating of P2B2T2, and PBT health testing is requested. Fate testing required is the FISH BCF (OECD: 305). Potential risks to the environment were high for both acute and chronic exposure. The company has already submitted acute Eco test data; therefore, only chronic testing is requested. Requested chronic tests are the continuous renewal method, analytical measurement of test substance for Fish Early Life-Stage (OPPTS: 850.1400) and Chronic Daphnia (OPPTS 850.1300). Continuous renewal method should be used due to the low solubility of the PMN.

[redacted]

[redacted]neration or Landfill: [redacted]/site-day over [redacted] days/yr from [redacted] sites

FATE: [redacted]
SWC: 607.55 ppb
DW: LADD: 1[redacted] mg/kg/d; ADR: [redacted] mg/kg/d

FATE: [redacted]
SWC: 22.64 ppb
DW: LADD: 1[redacted] mg/kg/d; ADR: [redacted] mg/kg/d
>COC (1 ppb): [redacted] d/yr

FATE: Stack Releases to Air
LADD: 3[redacted] mg/kg/d

P2 Rec Comments:

Testing:

Final Recommended:

Health: Potential health concerns resulted from the PBT rating of P2B2T2, and PBT health testing is requested.

Eco: The company has already submitted acute Eco test data therefore only chronic testing is requested. Requested chronic tests are the continuous renewal method, analytical measurement of test substance for Fish Early Life-Stage (OPPTS: 850.1400) and Chronic Daphnia (OPPTS 850.1300). Continuous renewal method should be used due to the low solubility of the PMN.

Fate: Fate testing required is the FISH BCF (OECD: 305)..

Other:

SAT Report

PMN Number: **P-09-0135**
SAT Date: **1/9/2009**
Print Date: **2/23/2015**

Related cases: [redacted]

Health related cases:
Ecotox related cases: Analogs: [redacted].

Concern levels:

Type of Concern:	**Health**	**Eco**	**Comments**
Level of Concern:	1-2	3	**Health: ; Eco:** chronic only

Persistence	**Bioaccum**	**Toxicity**	**Comments**
2	2	2 Awaiting Human Health Entry Awaiting Human Health Entry Awaiting Human Health Entry	

Exposure Based Review:

Health:
Ecotox: No

Routes of exposure:

Health: Dermal, inhalation
Ecotox: All releases to water
Fate: ;

Keywords:

Keywords:

Summary of Assessment:

Fate:

Fate Summary: P-09-0135

FATE:
Liquid with MP <20 EC (E)
log Kow = [redacted]
S = [redacted]

VP < [redacted]
BP = Dec. >220 EC (M)
H < 1.00E-8 (E)
POTW removal (%) = 90 via sorption and possible partial biodeg; OECD 310 (CO2 in sealed vessels): NRB/28 d.
Time for complete ultimate aerobic biodeg = mo
Sorption to soils/sediments = strong - v.strong
PBT Potential: P2B2
*CEB FATE: Migration to ground water = negl

Health:

Health Summary: Expect poor absorption via all routes (pchem). Concern for dermal sensitization based on submitted test data. Concern for liver toxicity and neurotoxicity based on [redacted] compounds. Concern for mutagenicity [(+) for sister chromatic exchange, (+) in a mouse lymphoma assay]; uncertain concern for developmental toxicity [8E-[redacted], LOEL = 160 mg/kg] and marginal concern for oncogenicity ([redacted]) based on [redacted] **Data:** (-) Salmonella with and without activation; (-) E. coli with and without activation; rat dermal LD0 = 2000 mg/kg; slight skin irritation in rabbits, cleared by 48 - 72 hours; mild eye irritation in rabbits; (+) for skin sensitization in a mouse local lymph node assay, EC3 = 23%

Ecotox:

Test Organism	Test Type	Test End Point	Predicted	Measured	Comments
fish	**96-h**	**LC50**	*	0.76	
daphnid	**48-h**	**LC50**	*	0.64	
green algal	**96-h**	**EC50**	*	0.56	0-72 hr EbC50
fish	**_**	**chronic value**	0.007		
daphnid	**_**	**chronic value**	0.014		
algal	**_**	**chronic value**	0.016		or *
Sewage Sludge	**3-h**	**EC50**	**_**		
Sewage Sludge	**_**	**Chronic Value**	**_**		

Ecotox Values Comments:

Factors	Values	Comments
Assessment Factor	10	
Concentration of Concern (ppb)	6	
SARs		

SAR Class		
Ecotox Category		

Ecotox Factors Comments:

SAT Chair: L Keifer 564-8916

1

INITIAL REVIEW ENGINEERING REPORT [redacted]

P-09-0135
Focus Ready Draft 1/15/2009

ENGINEER: Arnold \ JAS
PV (kg/yr): [redacted]

Revision Notes/Assessment Overview:

SUBMITTER: ICL-IP America Inc. (submitter)

USE: Flame retardant for [redacted]. There is one reference in file CA on [redacted], it is for use as a flame retardant for [redacted] Analog [redacted] is a flame retardant for [redacted].

OTHER USES: [redacted] is a chemical [redacted] that is used to [redacted]).

MSDS: Yes **LABEL**: No

Gen Eqpt: protective gloves / chemical safety goggles / body covering and boots / provide adequate ventilation.
Respirator: approved respirator
Health Effects: mild eye irritant
TLV/PEL: - none established
LVE PPE:

CRSS: ([redacted] AM):
Chemical Name: [redacted]
S-H2O: [redacted]
VP: [redacted]
[redacted]
Physical State and Misc CRSS Info:
Neat: Liquid **Mfg:** NK - Import
Proc/Form: [redacted] **End Use** [redacted]
[redacted].

[redacted]

SAT (concerns): (1/9/2009):

Migration to groundwater:
PBT rating: P2 **B**2 **T**2
Health: 1-2, Dermal, Inhalation
Eco: 3, Water (All releases to water with a CC = 1 ppb)

OCCUPATIONAL EXPOSURE RATING: 1B

NOTES & KEY ASSUMPTIONS:
Generated by the 06/07/2005 version of ChemSTEER. This IRER is for a PMN is used as a flame retardant for [redacted], with a PV of [redacted] kg/yr. The PMN is import only; therefore MFG is not assessed. Technical contact was called; see contact report. The SAT report lists dermal/inhalation exposures; and releases to water with a concentration of 1 ppb as concerns. [redacted]

required, so all exposures and releases are considered. [redacted]

P2 REC:

EXPOSURE-BASED REVIEW: [redacted] criteria met)

P-09-0135

Use: Polyurethane Foam

Number of Sites/Location: [redacted] submitter site(s)
unknown site(s)
Basis: Submission estimates use at [redacted] sites. Technical contact indicates PMN is imported at [redacted] concentration. No number of days or batch size is provided. CEB assumes [redacted] operating days. ChemSTEER calculates batch size of [redacted] kg PMN/batch.
Process Description: [redacted]

ENVIRONMENTAL RELEASES ESTIMATE SUMMARY

IRER Note: The daily releases listed for any source below may coincide with daily releases from the other sources to the same medium.

Water or Incineration or Landfill
High End: 6[redacted] kg/site-day over [redacted] day/yr from [redacted] sites or [redacted] kg/yr
to: uncertain
from: [redacted]

Water or Incineration or Landfill
Conservative: [redacted] /site-day over [redacted] day/yr from [redacted] sites or [redacted] kg/yr
to: uncertain
from: [redacted]
.

RELEASE TOTAL

[redacted] /yr - [redacted]

OCCUPATIONAL EXPOSURES ESTIMATE SUMMARY

Tot. # of workers exposed via assessed routes: [redacted]
Basis:

Dermal:
Exposure to imported liquid PMN is assessed below. Exposures to final foam product are not expected, because PMN [redacted], per CRSS.

Exposure to Liquid
High End: 1[redacted] mg/day over [redacted] days/yr
Number of workers (all sites) with Dermal exposure: [redacted]
Basis: [redacted]

Exposure Report

PMN Number: **P-09-0135**
2/23/2015 2:27:05 PM

Assessor: Cinalli

Ratings
Non-Occupational: 1 Environmental: 2

Drop Criteria:
Criteria Reasoning:

Exposure Based Assessment:

Exposure-Based (Non-Occupational):
Exposure-Based (Environmental):

Exposure Parameter	Exposure-Based	Persistent/Bioaccum	Exposure Value
Surface DW:		No	
Fish Ingestion:		No	
Ground DW:		No	
Inhalation:		No	
Water Releases:		No	
Total Releases:		No	
Consumer Exposure:		No	

Recommended Fate Tests:

Consumer Use expected? [redacted]
Consumer Use remarks:

	Activity	Scenario	Sites	Location

**Environmental Release Information for Those Releases Which Went to the Model (Release Identifier Number)

1